

REMARKS

A. Status of the Claims

Claim 67 was pending and rejected in the final Office Action dated February 12, 2002. In this Preliminary Amendment, Applicants add claims 86-89. Support for the added claims may be found throughout the specification at least at the following locations: page 8, lines 25 to page 9, line 4; page 9, lines 6-12; page 9, lines 21-23; page 14, lines 19-23; page 15, lines 1-5; and, page 16, lines 5-10. Accordingly, no new matter has been added. A copy of the pending claims in this case can be found in Appendix A.

B. Provisional Obviousness-Type Double Patenting Rejection

The Action provisionally rejects claim 67 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 22, 29, and 32-34 of co-pending Application No. 09/668,532. Once the present claims are in condition for allowance, Applicant will submit, if appropriate, a terminal disclaimer.

C. Factual Evidence Proves Claims Are Described

The Action rejects claim 67 as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Action argues that the "present specification provides no such reasonable clarity to those skilled in the art that applicant was in possession of the claimed invention." Action at page 3. Applicant respectfully traverses this rejection.

The claims are generally directed to an "adenovirus vector comprising a wild type p53 gene under the control of a promoter." The specification makes clear that the inventor was in possession of the claimed invention:

- “In one specific embodiment, the invention concerns vector constructs for introducing wild type p53 genes (wt-p53) into affected target cells suspected of having mutant p53 genes. These embodiments involve the preparation of a gene expression unit wherein the wt-p53 gene is placed under the control of the β -actin promoter, and the unit is positioned in a reverse orientation into a retroviral vector.” Specification at page 9, lines 6-12.
- In Example III, “The p53 cDNA with its β -actin promoter was cloned into the LNSX retroviral vectors in *both* orientations.” Specification at page 61, lines 29-30 (emphasis added).
- “While this affect [sic] was observed using the β -actin promoter and a retroviral expression vector, the inventors believe that this phenomenon *will be applicable to other promoter/vector constructs for application in gene therapy.*” Specification at page 8, line 25 to page 9, line 4 (emphasis added).
- “In addition to retroviruses, it is contemplated that *other vectors can be employed, including adenovirus...*” Specification at page 14, lines 21-23 (emphasis added).
- “While the β -actin promoter is preferred the invention is by no means limited to this promoter and one may also mention by way of example promoters derived from RSV, N2A, LN, LNSX, LNSN, SV40, LNCX or CMV.” Specification at page 15, lines 1-4 (citations omitted).
- “*Generally speaking*, such a promoter might include either a human cellular or viral promoter. While the β -actin promoter is preferred the invention is by no means limited to this promoter...” Specification at page 14, line 35-page 15, lines 2 (emphasis added).
- “While the retroviral construct aspect of the invention concerns the use of a β -actin promoter in reverse orientation, there is no limitation on the nature of the selected gene which one desires to have expressed. Thus, the invention concerns the use of antisense-encoding constructs *as well as ‘sense’ constructs that encode a desired protein.*” Specification at page 16, lines 5-10.

Therefore, the Specification makes clear that 1) p53 sense constructs are contemplated in both orientations; 2) any discussion about antisense constructs applies to “sense” constructs such as p53; 3) constructs can be retroviral, but they may also be adenovirus constructs; 4) promoters are discussed both generally and in the context of antisense constructs, in addition to CMV being specifically mentioned; and finally, 5) since an adenovirus can be used instead of retrovirus and

since constructs are not limited to antisense constructs, applying equally to sense constructs, there is adequate written description for an “adenovirus vector comprising a wild type p53 gene under the control of a promoter,” as well as for vectors with a CMV promoter.

In addition to the Declaration of Dr. Lou Zumstein, submitted with the Response filed on October 18, 2001, Applicant submits the Declaration of Dr. Philip Hinds (Appendix B). Both of these constitute evidence from a person of ordinary skill in the art to support the contention that the applicant was in possession of the claimed invention at the time the priority application was filed. Applicant contends that the Action has not rebutted the evidence submitted by persons of ordinary skill in the art to maintain the rejection of these claims. Evidence, as opposed to examiner argument, should be required to meet the “preponderance of the evidence” standard set forth in MPEP § 2163.04. The declarations and the identified portions of the specification show the written description requirement has been met. Accordingly, Applicant respectfully requests this rejection be withdrawn.

D. Claims Are Enabled

The Action rejects claim 67 under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention. The Action contends that the specification does not provide a source for packaging cell lines for the production of adenoviral vectors that contain the p53 gene in a region of the adenoviral genome essential for replication. It further argues that the specification nor the art provide a source for packaging cells that complement deletions in different regions of adenovirus, and consequently, that the breadth of the claim is not enabled. Applicant traverses this rejection.

The art cited in the Action supports, rather than disputes, the required assumption that the specification is enabling. Satisfaction of the enablement requirement is not precluded by the necessity of some experimentation. See *Atlas Powder Co. v. E.I. duPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409 (Fed. Cir. 1984). The test of enablement is whether the experimentation needed to practice the invention is **undue**. MPEP § 2164.01 (citing *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916)).

The Action cites two references to argue that some experimentation is required to generate different adenovirus complementing cell lines. The Action relies on U.S. Patent 5,994,106, which the Action contends supports its contention that “a summary of the relevant art taught that complementing or packaging cell lines for other than adenovirus E1 were not in use.” However, neither the cited patent, nor the other cited reference of Armentano, shows that the amount of experimentation is undue. In fact, instead of showing the invention is inoperable or reads on inoperable species, Armentano and other references indicate that complementing cell lines other than 293 cells were made and that other such cell lines could be made. As the Action notes, Armentano *et al.* acknowledges that E2 (E2A)-complementing and E4-complementing cell lines were made; this statement is based on two references, Brough *et al.* 1992 (Appendix C) and Weinberg *et al.* 1983 (Appendix D), which were published prior to the filing of the present application. Another article by Rice *et al.* (1985) (Appendix E) further confirms the availability of E2A-complementing cell lines. Thus, there is proof that there were other cell lines available. The Action’s contention that cell lines other than 293 cells were unavailable at the time the application was filed is inaccurate. As such, there is no *prima facie* basis to reject the claims as not enabled.

Furthermore, the enablement rejection is misplaced to the extent that the Action generally contends that the application has not enabled second and third generation adenovirus vectors. “A patent applicant is not required . . . to predict every possible variation, improvement or commercial embodiment of his invention.” *United States Steel Corp. v. Phillips Petroleum Co.*, 673 F. Supp. 1278, 1292 (D. Del. 1987), *aff’d*, 865 F.2d 1247, 1250 (Fed. Cir. 1989) (specifically quoting this statement). This is consistent with the Federal Circuit’s view of later developed technology. The mere fact that a claim may be found to cover after-developed technology does not in itself lead to a conclusion that the claim is invalid for failing to enable the claimed technology. *Hormone Research Found., Inc. v. Genentech, Inc.*, 904 F.2d 1558, 1568 (Fed. Cir. 1990). The issue of enablement is whether the specification enables one skilled in the art to practice the claimed invention *at the time the application is filed*. *United States Steel Corp.*, 865 F.2d at 1251.

The claimed invention was enabled at the time the application was filed. Based on the foregoing evidence and arguments, Applicant respectfully requests this rejection be withdrawn.

E. Claims Are Not Obvious

The Action rejects claim 67 under 35 U.S.C. § 103 (a) as being unpatentable over Chen *et al.* (1990) (Chen) in view of Colicos *et al.* (1991) (Colicos) further in view of Pasleau *et al.* (1986) (Pasleau). The Action contends that “the motivation by Chen was to use a vector comprising p53 to revert the transformed phenotype, the motivation to use Colicos was that adenovirus has a broad host range for the study of mammalian gene expression and the motivation to use Pasleau was that the CMV promoter had exhibited strong expression levels.” Applicant respectfully traverses this rejection.

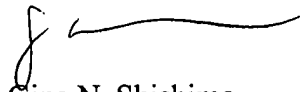
A *prima facie* case of obviousness requires that a suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to **modify the reference** or to **combine reference teachings**. MPEP § 2143. The purported “motivation statements” in the Action do not satisfy this requirement. Instead, the motivation statements simply identify what the cited articles teach. The Action does not, however, indicate any reason why a person of ordinary skill in the art would combine or modify the references—that is, why a person of ordinary skill in the art would create an adenovirus vector comprising a wild-type p53 gene under the control of a promoter or specific promoters. Why would a person modify the Chen reference to employ the adenovirus vector taught by Colicos? It is not convincing or sufficient to answer that a person would do it because Colicos teaches adenovirus has a broad host range for the study of mammalian gene expression as this simply begs the question: why would a person of ordinary skill in the art want a broad host range for the study of mammalian gene expression in the context of Chen’s showing that p53 reverts the transformed phenotype? Why would a person modify the Chen reference to employ an adenovirus when Chen shows that its retrovirus vector accomplishes the results set forth in the paper? Alternatively why would a person of ordinary skill in the art replace the *denV* gene from bacteriophage T4 with a wild-type p53 gene? That Chen shows p53 reverts the transformed phenotype or that Colicos shows adenovirus has a broad host range for the study of mammalian gene expression simply do not answer that question. In fact, none of the references can. The question of “Why produce the claimed invention?” goes to the heart of the motivation/suggestion requirement. Without a sufficient answer, none of the references provides the requisite motivation or suggestion to produce the claimed invention. Accordingly, a proper *prima facie*

case of obviousness has not been made. Applicant respectfully requests this rejection be withdrawn.

F. Conclusion

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/10012299/GNS.

Respectfully submitted,



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Attorney for Applicants

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Date: May 13, 2002

APPENDIX A:
PENDING CLAIMS

67. (Amended) An adenovirus vector comprising a wild type p53 gene under the control of a CMV promoter.
86. An adenovirus vector comprising a wild type p53 gene under the control of a promoter.
87. The vector of claim 86, wherein the promoter is the β -actin promoter.
88. The vector of claim 86, wherein the promoter is the SV40 promoter.
89. The vector of claim 86, wherein the promoter is the RSV promoter.



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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Jack A. Roth

Serial No.: 09/447,681

Filed: November 23, 1999

For: ADENOVIRUS p53 COMPOSITIONS
AND METHODS

Group Art Unit: 1632

Examiner: Crouch, D.

Atty. Dkt. No.: INRP:003--2

DECLARATION OF PHILIP W. HINDS, PH.D UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Philip W. Hinds, Ph.D., declare the following:

1. I am an Associate Professor in the Pathology Department at Harvard Medical School. I have a Ph.D in Molecular Biology from Princeton University. My doctoral dissertation focused on p53 and its role as an anti-oncogene. At Harvard, I have done research on tumor suppressors, including p53 and Rb. I have authored a number of scientific papers on these topics. My *curriculum vitae* is attached as Exhibit 1. Based on my knowledge of the scientific literature and my own research, I am familiar with the

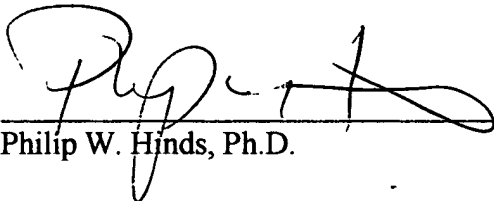
level of skill of a person with ordinary skill in the p53 field in the 1992 to 1993 time frame.

2. I have reviewed the specification of the instant application.

3. It is my opinion that the specification indicates that the inventors contemplated that adenovirus could be substituted for retrovirus as a vector for wild-type p53 under the control of a promoter. I base this conclusion on the wording of the specification, which makes it clear that the inventors envisioned and planned for an adenovirus vector encoding wild-type p53 under the control of a promoter. In particular, I base my conclusions on page 9, lines 6-8 and page 14 lines 21-23 of the specification.

4. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

3 May 2002
Date


Philip W. Hinds, Ph.D.

Principal Investigator/Program Director (Last, first, middle):

Hinds, Philip W.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. DO NOT EXCEED FOUR PAGES.

NAME

Philip W. Hinds, Ph.D.

POSITION TITLE

Associate Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Maine, Orono, Maine	BS	1983	Biochemistry
University of Maine, Orono, Maine	MS	1984	Biochemistry
Princeton University, Princeton, New Jersey	Ph.D.	1989	Molecular Biology
Whitehead Institute, Cambridge, MA	postdoc	1993	Molecular Biology

Professional Experience

- 1993 Instructor of Biochemistry, University of Maine, Department of Biochemistry
- 1985-1989 Ph.D. Molecular Biology (October 1989), Princeton University, Princeton, NJ 08544. Thesis advisor: Arnold J. Levine, Ph.D.; Project: Function of the p53 oncogene/tumor suppressor
- 1989-1993 Postdoctoral Fellow, Whitehead Institute, Cambridge, Massachusetts
Laboratory advisor: Robert A. Weinberg, Ph. D.
- 1993-2000 Assistant Professor, Department of Pathology, Harvard Medical School, Boston, MA
- 1997-2000 American Cancer Society Cell Cycle Study Section, Chair 1998-2000
- 2000- Associate Professor, Department of Pathology, Harvard Medical School, Boston, MA

Honors and Awards

- 1983 Frederick H. Radke Scholar in Biochemistry (Department of Biochemistry, University of Maine)
- 1984 George F. Dow Award (College of Life Sciences and Agriculture, University of Maine)
- 1985 Baxter-Travenol Fellowship
- 1986 U.S. Public Health Service National Research Service Award CA09528
- 1988-1989 New Jersey Commission on Cancer Research Fellowship.
- 1989-1992 Postdoctoral Fellowship (Leukemia Society of America)
- 1992-1993 Sokol Scholar (Whitehead Institute)
- 1992-1993 Ladies Auxiliary to the Veterans of Foreign Wars Postdoctoral Fellowship
- 1993-1995 Harcourt General Charitable Foundation Research Grant
- 1994 American Cancer Society (Massachusetts Division) Research Grant
- 1994-1998 U.S. Army Junior Faculty Breast Cancer Research Grant #DAMD17-94-J4258
- 1995-2001 American Cancer Society Research Grant
- 1995-2000 Scholar, Leukemia Society of America
- 1997-2002 NIH Research Grant 1R01GMCA55684-01

Publications (Partial listing)

- Hinds, P.W., Finlay, C.A., Frey, A.B. and Levine, A.J. (1987). Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. Mol. Cell Biol. 7: 2863-2869.
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Principal Investigator/Program Director (Last, first, middle): Hinds, Philip W.

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- Marks, J.R., Lin, J., Hinds, P., Miller, D. and Levine, A.J.** (1989). Cellular gene expression in papillomas of the choroid plexus from transgenic mice expressing the SV40 large T antigen. *J. Virol.* 63: 790-797.
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- Maren, M.C., Jost, C.A., Brooks, L., Irwin, M.S., O'Nions, J., Tidy, J.A., James, N., McGregor, J.M., Harwood, C.A., Yulug, I.G., Vousden, K.H., Allday, M.J., Gusterson, B., Ikawa, S., Hinds, P.W., Crook, T. and Kaelin Jr., W.G.** (2000). A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. *Nature Genetics* 25: 47-54.
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Principal Investigator/Program Director (Last, first, middle): Hinds, Philip W.

Reviews

Tiemann, F., Musunuru, K., and Hinds, P.W. (1997). The Retinoblastoma Tumour Suppressor Protein and Cancer. In: *Prot. In Dysfunction in Human Genetic Disease.*, Y. Edwards and D. Swallow, eds. Bios Scientific Publishers, Oxford. pp. 163-185.

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Research Support

Hinds, P.W.

ONGOING

1 R01 GM/CA55684 Hinds (PI)	5/01/1997-4/30/2002	30%
NIH GM/NCI	\$161,476	
Proliferative effects of cdk4 and cdk6 dysregulation		

The overall goal of this project is to identify the mechanisms by which cdk4 and cdk6 contribute to the tumorigenic phenotype with particular emphasis on discrete roles for these kinases in different cell types.

1 R01 AG/CA20208 Hinds (PI)	2/01/2002-1/31/2007	30%
NIH NIA/NCI	\$200,000	
Role of pRb in osteogenesis, cell cycle exit and cancer		

The goal of this project is to explore the mechanism through which pRb activates CBFA1-dependent transcription in particular and the role of pRb in bone differentiation in general. In addition, mechanisms of pRb action in cell cycle exit in senescence and differentiation are investigated.

1P01 DE12467 Wong (PI)	04/01/1998-03/31/2003	10%
NIH/NIDCR	\$131,308	
Cell Cycle Regulators of Oral Cancer		

The overall goal of this program project is to advance understanding of the molecular basis of oral cancer. Project one, "HPV and Cell Cycle Dysregulation in Oral Cancer", has as its goal the elucidation of the mechanisms of loss of cell cycle control in oral epithelial tumors. The budget is split equally with Karl Mûnger, who is co-PI of this project.

COMPLETED

RPG-95-013-04-CSM	01/01/1998-12/31/1999
American Cancer Society	
Function of the Retinoblastoma Protein and D-Cyclins in Cancer	

The goal of this project was to discriminate among specific roles of the retinoblastoma protein in cancer, differentiation and senescence. The role of cyclin D1 in development and cancer was an early goal of this project that is now the subject of ongoing work.

RPG-95-013-06-CCG Hinds (PI)	01/01/2000-12/31/2001
American Cancer Society	\$100,000
Function of pRb in Differentiation, Senescence and Cancer	

The goal was to understand the role of pRb in terminal cell cycle exit at the cellular and organismal level.

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Construction, Characterization, and Utilization of Cell Lines Which Inducibly Express the Adenovirus DNA-Binding Protein

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To further our understanding of structure-function relationships within the multifunctional adenovirus DNA binding protein (DBP) a more diverse collection of mutants is necessary. DBP-expressing cell lines (gmDBP) were previously constructed that complemented DBP-negative mutants for viral growth. However, they did not allow severely defective viruses to form plaques. Since efficient mutant construction is reliant on plaque isolation of the desired mutant virus as a final step, additional gmDBP cell lines were constructed which allow all DBP-negative mutants to form plaques. Here we describe the construction and characterization of 12 new gmDBP cell lines. The utility of these lines was demonstrated by the efficient construction of a new defective mutant, H5in804, using a combination of DBP-expressing lines. The H5in804 mutation adds 22 amino acids at the carboxyl end of an otherwise wild type protein. Characterization of H5in804 revealed that it was altered in its ability to replicate viral DNA. The depression of DNA synthesis most probably results from a reduced ability of H5in804 DBP to bind ssDNA. © 1992 Academic Press, Inc.

INTRODUCTION

The adenovirus ssDNA binding protein (DBP) is an excellent example of a multifunctional protein. Mutants have been invaluable in discerning relationships between structure and function for DBP and describing the multiple roles played by DBP throughout the course of an adenovirus infection. Characterization of mutants with temperature sensitive (ts) lesions in the DBP gene (especially the prototype DBP mutant, H5ts125) have suggested a myriad of functions played by DBP. DBP is required for viral DNA replication at several different phases of this complex process (Horwitz, 1990; van der Vliet, 1990; Williams and Chase, 1990; Brough *et al.*, 1992). Studies on these ts mutants and their revertants have further suggested that DBP plays a role in the control of early gene expression (Carter and Ginsberg, 1976; Carter and Blanton, 1978a,b; Blanton and Carter, 1979; Nevins and Winkler, 1980; Babich and Nevins, 1981; Nicolas *et al.*, 1982), morphological transformation (Ginsberg *et al.*, 1974; Rubinstein and Ginsberg, 1974; Williams *et al.*, 1974), and virion assembly (Nicolas *et al.*, 1983).

Another type of DBP mutant (host range, hr) allows human adenovirus to grow productively on monkey cells (Klessig, 1977; Anderson, 1981). Wild type (wt) adenovirus is blocked for growth on monkey cells because late gene expression is altered (Klessig and Anderson, 1975; Anderson and Klessig, 1982). The block to late gene expression, which the hr mutations over-

come, is due to reduced transcription of late genes (Johnston *et al.*, 1985), an altered pattern of RNA splicing of the L1 and L5 late regions (Anderson and Klessig, 1984; Anderson *et al.*, 1988), and poor utilization of the fiber (L5) mRNA in abortively infected monkey cells (Silverman and Klessig, 1989).

The hr mutations (aa 130) (Klessig and Grodzicker, 1979; Kruijer *et al.*, 1981; Anderson *et al.*, 1983; Brough *et al.*, 1985) and an additional mutation which allows the hr virus to overcome cold sensitivity in monkey cells (aa 148) (Brough *et al.*, 1985) are located in the amino-terminal (N-t) portion of DBP. The ts mutations that alter ssDNA binding and DNA replication (aa 280, 282, and 413) (van der Vliet *et al.*, 1975; Kruijer *et al.*, 1981, 1982; Prelich and Stillman, 1986) are localized within the carboxyl-terminal (C-t) portion. Analysis of H2ts400 which contains both hr and ts mutations indicates that the N-t and C-t regions of the protein function independently (Rice and Klessig, 1984; Brough *et al.*, 1985). Additional evidence that the protein contains at least two structurally and functionally distinct domains includes sequence comparison of the different serotypes (C-t is highly conserved while the N-t is not) (Quinn and Kitchingman, 1984; Kitchingman, 1985; Vos *et al.*, 1988), different amino acid contents of the two regions, and cleavage of the protein by a variety of proteinases which results in two distinct fragments (Klein *et al.*, 1979; Linne and Philipson, 1980; Schechter *et al.*, 1980; Tsernoglou *et al.*, 1985). The N-t domain is responsible for the control of late gene expression while the C-t domain is responsible for binding to nucleic acids and functioning in DNA replication.

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The development of cell lines expressing wt DBP (gmDBP1-5) (Klessig *et al.*, 1984a) facilitated construction of site-directed mutants within the DBP gene. The first set of directed mutants constructed contained large deletions which essentially eliminate DBP expression (Rice and Klessig, 1985). The prototype for this set, H5dl802, demonstrates an absolute requirement of DBP for viral DNA replication and suggests that DBP's control of E1 and E4 expression and morphological transformation are properties of the mutated H5ts125 protein and not properties of the wt protein (Rice and Klessig, 1985; Rice *et al.*, 1987). Revertants of H5dl802, exemplified by H5dl802r1 (Cleghon *et al.*, 1989), show the importance of the N-t region in the efficient nuclear localization of DBP. Subsequent site-directed mutants within the N-t region defined two motifs necessary for nuclear transport (Morin *et al.*, 1989). Other sets of directed mutants within the N-t domain of DBP further implicate this region in the control of E2A expression (Rice and Klessig, 1985; Cleghon *et al.*, 1989; Morin *et al.*, 1989) and a DNA replication function distinct from that performed by the C-t domain (Brough *et al.*, 1992).

Directed DBP mutants have provided much insight into our understanding of structure-function relationships. However, we are still limited in our knowledge of DBP due to the lack of sufficient mutants throughout the DBP gene. The previously constructed gmDBP cell lines which inducibly express wt DBP complement nonviable DBP mutant viruses for growth, but do not allow the viruses to form plaques. The lack of plaque formation on these cell lines makes mutant virus construction and isolation extremely difficult and time consuming. An insufficient level of DBP was thought to be responsible for the failure of these lines to facilitate plaque formation by the most severely debilitated DBP mutants. Therefore, another set of gmDBP cell lines was produced that contained the SV40 enhancer to increase DBP expression. Here we describe the construction and characterization of these new gmDBP cell lines which allow even severely defective DBP mutants to form plaques. The utility of these lines is demonstrated by the efficient construction of a virus carrying a DBP mutation that is lethal for the growth of the virus.

MATERIALS AND METHODS

Cells and cell transformation

The parental HeLa cell line (provided by J. F. Williams), as well as the gmDBP lines passaged in the absence of selection for *Escherichia coli* xanthine-guanine phosphoribosyltransferase (gpt) expression, were grown on Dulbecco's modified Eagle's medium

(Flow Labs) supplemented with 10% calf serum, 100 μ g per ml streptomycin, 100 μ g per ml penicillin, and 292 μ g per ml glutamine. Propagation, transformation, selection, and isolation of transformants expressing the (gpt) gene were as described in Klessig *et al.* (1984a). To remove the gmDBP cell lines from selection the monolayers were first allowed to reach 50% confluency and the media was changed to normal growth conditions. Maintenance of a high density was necessary for several passages to avoid a lag period which occurred if the lines were removed from selection at low cell densities.

Construction of pMSG-DBP-EN

A fragment containing two copies of the SV40 72-bp enhancer, a 164-bp *Bam*HI fragment from pACT (gift of M. Botchan) was isolated from a 5% polyacrylamide gel by diffusion. After organic extractions and ethanol precipitation the overhanging *Bam*HI ends were blunt ended with Klenow and *Cla*I linkers were added. The *Cla*I-modified fragment was ligated into *Cla*I-linearized pMSG-DBP (Klessig *et al.*, 1984a) that had been treated with calf intestinal phosphatase (Boehringer-Mannheim). The resulting construct, pMSG-DBP-EN was verified by restriction enzyme analyses. Unless otherwise stated all reagents were supplied by New England Biolabs and techniques were as described in Maniatis *et al.* (1982).

Screening and analyses of gmDBP cell lines

Levels of DBP accumulation in confluent monolayers of the new gpt⁺ cell lines (approximately 3×10^6 cells per 60-mm dish) after 24 hr of dexamethasone treatment were compared to the level of DBP expressed in the dexamethasone-induced gmDBP2 cell line (Klessig *et al.*, 1984a). Aliquots of each cell lysate were immunoprecipitated with polyclonal anti-DBP, the proteins fractionated by SDS-PAGE (10% polyacrylamide gel) and transferred to nitrocellulose. Immunoblot detection of DBP was as previously described except that 5% nonfat milk was used as a blocking agent, a 1:75 dilution of polyclonal anti-DBP was used, and reactions were done at room temperature in a plastic container (Klessig *et al.*, 1984b). Cellular DNA preparation, single-copy Southern analysis, and the kinetics of DBP synthesis and accumulation in gmDBP cell lines was as described in Klessig *et al.* (1984a).

To screen for the ability to support virus plaque formation the lines were first removed from gpt⁺ selection and passaged at least 10 generations. When grown in the presence of gpt⁺ selection none of the lines supported wt Ad5 plaque formation. Plaque assays were performed as described in Rice and Klessig (1985) with

the addition of 6×10^{-7} M dexamethasone to all overlays. A cell density of approximately 85–90% confluency at the initiation of plaque assays was important to maintain excellent plaque formation. Under these optimum conditions, gmDBP2 supported H5dl802 plaque formation to known titers only one out of 10 times, while gmDBP6 reproducibly gave consistent titers.

Construction, propagation, and analysis of a lethal DBP mutant

The *Cla*I linker insertion mutation at the *Dra*I site in the DBP gene was previously described as a lethal DBP mutation (Vos *et al.*, 1989). This mutation alters the reading frame of the DBP gene at the termination codon and effectively adds 22 amino acids to the C-end of the otherwise wt protein. The virus construction method used was the *in vivo* single-recombination procedure described in Vos *et al.* (1989) and Morin *et al.* (1989). Virus plaque isolation, propagation, and analysis of virus growth properties were as previously described (Klessig, 1977; Rice and Klessig, 1985).

Viral DNA was isolated by a modified Hirt procedure (Hirt, 1967; Rice and Klessig, 1984). DNAs purified by Hirt extraction were digested with *Eco*RI and separated on a 0.8% agarose gel. Viral DNA accumulation was determined directly by ethidium bromide staining of the gels. For quantitation of viral DNA synthesis, infected HeLa monolayers were labeled with [3 H]thymidine for 1 hr at the designated times postinfection before low-molecular-weight DNA was isolated. The *Eco*RI-digested and fractionated DNAs were transferred to nitrocellulose, fluorographed, and the amount of label incorporated into each band quantitated with a Joyce-Lobel Chromoscan 3. This value was then represented graphically as the percentage of peak wt Ad5 DNA synthesis.

The methods for determining synthesis and accumulation of DBP, pulse-chase experiments analyzing DBP stability, techniques used for *in situ* immunofluorescence microscopy, and the method used to analyze mutant DBP ssDNA binding ability have all been described previously (Anderson and Klessig, 1983; Klessig *et al.*, 1984b; Volkerding and Klessig, 1986; Brough *et al.*, 1992). Buffer AB contains 10 mM potassium phosphate, pH 6.8/1 mM EDTA/0.5 mM dithiothreitol/30 μ g per ml phenylmethylsulfonyl fluoride/7% glycerol, and the indicated concentration of NaCl.

RESULTS

Construction of DBP-expressing cell lines

The DBP gene contained within the plasmid construct pMSG-DBP is expressed from the dexametha-

sone (DM) inducible mouse mammary tumor virus (MMTV) promoter (Klessig *et al.*, 1984a). This vector also carries the *E. coli* xanthine-guanine phosphoribosyltransferase (*Eco*-gpt) gene which is expressed from the SV40 early promoter. *Eco*-gpt is a dominant selectable marker that allows cells expressing this gene to grow in the presence of mycophenolic acid (MPA) (Mulligan and Berg, 1981).

Cells stably transformed with pMSG-DBP (gmDBP1-5) were previously constructed and characterized (Klessig *et al.*, 1984a). The level of expression of DBP in these lines was sufficient to complement conditional lethal DBP ts mutants (Klessig *et al.*, 1984a) and nonviable DBP deletion mutants (Rice and Klessig, 1985) for virus growth. However, the nonviable DBP mutants were unable to form plaques on these gmDBP cell lines. Insufficient levels of DBP were thought to be responsible for this inability to form plaques.

To increase the level of DBP expression the SV40 enhancer was cloned into pMSG-DBP at a unique *Cla*I site in the MMTV promoter (see Materials and Methods for details). The resulting plasmid, pMSG-DBP-EN, depicted in Fig. 1 was used to construct new DBP-expressing cell lines. HeLa cells were transfected with 0.4–5 μ g of *Eco*RI-linearized pMSG-DBP-EN, and transformed cells were selected for growth in the presence of MPA. The colonies remaining after 3–4 weeks of growth on selection were clonally isolated and passaged to insure that they were stable with respect to gpt expression. Thirty-seven gpt⁺ cell lines were established from these independent gpt⁺ colonies.

Screening clones for DBP expression

The 37 gpt⁺ lines were analyzed for their ability to produce DBP. Expression of DBP required that the line was cotransformed with adenoviral sequences and that the DBP gene was active. Immunoblot analysis (summarized in Table 1) showed that 84% of the 37 gpt⁺ cell lines accumulated DBP. Transfections with pMSG-DBP, the construct without the SV40 enhancer, resulted in only 8.6% of the gpt⁺ cell lines coexpressing the DBP gene (Klessig *et al.*, 1984a). Therefore, transfection with pMSG-DBP-EN increased the efficiency of cotransformation and expression of the DBP gene by nearly 10-fold over that obtained with pMSG-DBP.

Thirteen of the 31 cell lines expressed DBP equal to or above the level produced in gmDBP2, the best DBP-expressing cell line previously constructed (data not shown). Eight of these 13 grew well both on and off selection, while 3 grew well only off selection. Two cell lines grew poorly both on or off selection, and one of these was discontinued.

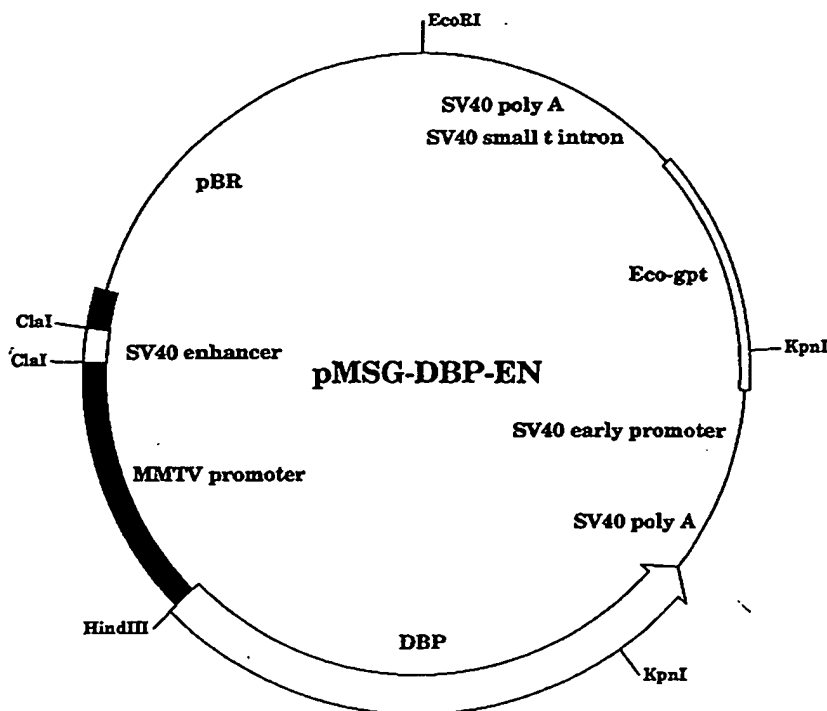


Fig. 1. Schematic representation of the pMSG-DBP-EN transformation vector. Pertinent sequences and restriction enzyme sites are denoted.

Screening of DBP expressing cell lines for plaque formation of H5dl802

The remaining 12 cell lines which accumulated high levels of DBP and formed adequate monolayers were further characterized for their ability to support plaque formation of wt and mutant viruses that carried deletions of the DBP gene. When the cell lines were grown on selection, i.e., in the presence of MPA, all failed to plaque wt virus. However, when the lines were taken off selection and passaged for greater than 10 generations several different phenotypes were obtained (Table 2). Two (22a1 and 22c1) of the 12 failed to plaque wt virus off selection and 2 (32h1 and 32h2) plaqued wt virus only inefficiently. The remaining 8 cell lines supported plaque formation by wt virus as well as HeLa monolayers. In fact, 4 (21f1, 22h1, 22h2, and 22h12) of the 8 cell lines allowed wt Ad5 to form more distinct plaques which started about 12–24 hr earlier than those formed on HeLa monolayers. But more importantly these 8 cell lines supported plaque formation of H5dl802 (Table 2), a nonviable deletion mutant that expresses no detectable DBP or fragment of DBP (Rice and Klessig, 1985). Four (21f1 = gmDBP6, 22d1 = gmDBP7, 32f1 = gmDBP8, and 32h2 = gmDBP9) of the 8 allowed H5dl802 to form plaques to within two-fold the level seen on HeLa monolayers coinfecting with H5dl802 and the helper virus H5dl434 (E1 deletion virus, from mu 2.6–8.7) (Klessig *et al.*, 1982).

The level of DBP accumulation for these four cell lines was determined to see if the ability to plaque H5dl802 correlated with significantly increased levels of DBP expression (Fig. 2). The level of accumulation of DBP was compared to that in gmDBP2 as a control. The gmDBP9 cell line significantly overexpressed DBP in comparison with gmDBP2. This line also produced a much higher level of DBP even in the absence of DM induction. The gmDBP6 and gmDBP8 cell lines expressed one- to fourfold enhanced levels of DBP as compared to gmDBP2. In contrast to these overexpressors, the gmDBP7 cell line accumulated less DBP than that of gmDBP2. This analysis suggests that the level of DBP accumulation was not the only factor determining the ability of these lines to support plaque formation by the nonviable DBP deletion mutants.

Characterization of gmDBP6

Because of the superior plaquing ability of gmDBP6, it was characterized in detail to determine the number and arrangement of the integrated pMSG-DBP-EN. Analysis of total cellular DNA from gmDBP6 by Southern blotting showed only a single band from *EcoRI* digestions (data not shown). The intensity of this band compared with reconstructions using DNA from untransformed cells mixed with varying amounts of pMSG-DBP-EN indicated that the adenoviral sequences were present in approximately one copy per

TABLE 1

ABILITY OF *gpt*⁺ CELL LINES TO EXPRESS DBP

Cell line ^a	DBP expression ^b	Cell line	DBP expression
21a1	+	31e1	+
21 1	+	31e2	+
21e2	—	21f1	+
21f1	+	31g1	+
21f2	+	31g2	+
21f3	+	31h1	+
22a1	+	32e1	+
22c1	+	32f1	+
22d1	+	32f2	+
22e1	+	32f3	—
22g1	+	32g2	+
22g2	—	32h1	+
22h1	+	32h2	+
22h2	+	4g1	—
31a1	+	4h1	+
31b1	+	4h12	+
31c1	+	5c1	—
31d1	—	5d1	+
		5d2	+

^a Cells were grown in the presence of mycophenolic acid to maintain selection.

^b Established monolayers were treated with DM for 24 hours, and the expression of the DBP gene detected by immunoblotting.

cell. Analysis of the *Cla*I/*Kpn*I digestion products showed that the adenoviral and *Eco*-*gpt* genes were intact and sequences were maintained in the same arrangement as in the plasmid (data not shown). There-

TABLE 2

ABILITY OF gmDBP CELL LINES TO SUPPORT PLAQUE FORMATION

Cell line	Virus yield (PFU/ml)	
	Ad5 (×10 ⁹)	H5dl802 (×10 ⁶)
HeLa	3.9	DNP ^a
HeLa + H5dl434	—	1.5
18p = gmDBP2	4.1	DNP
22a1	DNP	DNP
22c1	DNP	DNP
22h2	3.8	DNP
4h12	1.7	DNP
22h1	4.1	0.1
22h12	3.6	0.2
32h1	0.6	0.2
4h1	2.7	0.3
21f1 = gmDBP6	3.8	3.2
22d1 = gmDBP7	2.4	1.6
32f1 = gmDBP8	2.3	2.0
32h2 = gmDBP9	0.7	0.9

^a DNP, did not allow plaque formation.

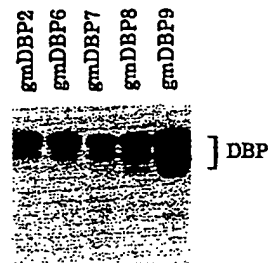


FIG. 2. Accumulation of DBP in the gmDBP cell lines which allowed DBP deletion viruses to form plaques. The amount of DBP was determined from confluent monolayers of each cell line grown in the absence of *gpt*⁺ selection and assayed after 24 hr of dexamethasone induction. DBP was immunoprecipitated with polyclonal anti-DBP from equal volumes of cell extracts, fractionated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose and subjected to immunoblotting using polyclonal anti-DBP. The amount of DBP produced in gmDBP2 cells treated under similar conditions was used as a control.

fore, the arrangement and copy number of the inserted gene in the gmDBP6 cell line is similar to that found in the well characterized DBP-expressing cell line, gmDBP2.

The amount of DBP produced in gmDBP6 at various times after induction with DM was compared to the amount produced in the gmDBP2 cell line. The level of DBP synthesis in a 1-hr pulse labeling with [³⁵S]-methionine at 0, 8, or 24 hr postinduction with DM was similar in gmDBP6 and gmDBP2 (data not shown). In both cell lines a minimal amount of synthesis was detected at 0 hr postinduction. The synthesis level was highest at 8 hr postinduction (approximately a 50- to 100-fold increase above that at 0 hr) and was only two-fold reduced from this peak level at 24 hr postinduction. The level of DBP synthesis at 8 hr post DM induction was equal to approximately 20% of that seen at the peak of DBP synthesis (24 hr p.i.) in adenoviral-infected cells.

Accumulation of DBP was followed in both gmDBP6 and gmDBP2 before and after induction with DM (Fig. 3). Even though the level of DBP synthesis was similar in the two cell lines, the level of DBP accumulation was two- to fourfold higher in gmDBP6 than in gmDBP2 after 24 hr of DM induction. A higher level of DBP accumulation was also seen in the absence of induction in gmDBP6. Since the level of synthesis in the two cell lines was comparable even in uninduced cells, the increase in accumulation of DBP must be due to increased stability of the protein in gmDBP6. The level of DBP accumulated 8 hr post DM induction in gmDBP6 and 24 hr postinduction in gmDBP2 was similar to the level of DBP that accumulated in Ad2-infected HeLa cells at 16 hr postinfection.

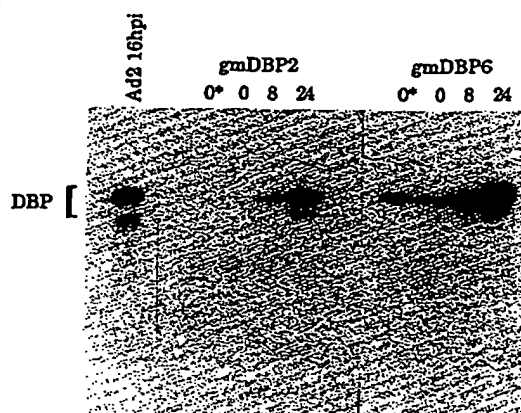


FIG. 3. The kinetics of accumulation of DBP in gmDBP6 was compared to gmDBP2 before and after induction with DM. Cells were grown off gpt⁺ selection and treated with 3×10^{-7} M DM for the indicated time in hours. Equal amounts of total cell lysate were immunoprecipitated with polyclonal anti-DBP, fractionated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose, and subjected to immunoblotting using polyclonal anti-DBP. The amount of DBP that accumulated 16 hr p.i. in Ad2-infected HeLa cells treated in a similar manner was used as a control.

Construction of mutant virus

To determine the utility of gmDBP6 for construction, as well as for propagation, of lethal DBP mutant viruses, we attempted to introduce a previously defined lethal mutation of DBP into the adenoviral genome and propagate it. Lethality of a DBP mutation was defined as the inability of the mutation to be constructed into virus using either 293 or HeLa cell lines. The mutation chosen, which had previously been found to be lethal (Vos *et al.*, 1989), contained a *Cla*I linker insertion in the stop codon (*Dra*I site) of the DBP gene. This insertion changes the reading frame at the stop codon resulting in the addition of 22 amino acids to the C-t of DBP.

Our method of virus construction relies on a single *in vivo* recombination within the overlapping regions between the left and right fragments of the genome (Morin *et al.*, 1989; Vos *et al.*, 1989). The left fragment of the genome (mu 0–59.5) is provided from *Bam*HI- and *Eco*RI-digested Ad5 DNA-terminal protein complex. The right genome fragment (45.9–100) which contains the mutated DBP gene is provided from a plasmid construct, such as p_{del}XE (pUC plasmid that contains adenoviral sequences from mu 46–100, and a deletion from *Xho*I to *Eco*RI, mu 83–84). Both fragments are introduced into the cell by transfection, and a mutant DBP virus is generated by the high frequency of adenoviral recombination.

The efficiency of transfecting intact adenoviral DNA-terminal protein complex varied dependent on the cell line transfected (Table 3). Using a modified cal-

cium phosphate transfection technique (Rice and Klesig, 1985) 293 cells yielded approximately 10^8 PFU per μ g of transfected Ad5 DNA-protein complex, while gmDBP2 gave only 6×10^3 PFU per μ g. The latter level was comparable to that found in transfections of HeLa cells (data not shown). However, the gmDBP6 cell line was at least 10^4 – 10^5 -fold lower in transfectability than either the parental HeLa line or gmDBP2. Because of this low level of transfectability, gmDBP6 could not be used for this stage of virus construction. The reason for differences in transfectability among DBP-expressing cell lines is not known.

Construction of virus was attempted utilizing 293 or gmDBP2 for transfection and HeLa or gmDBP6 for plaque isolation (Table 3). Construction of wt virus using the p_{del}XE vector, which contained a wt DBP gene, occurred at a frequency of 2×10^5 PFU per μ g of restriction-enzyme-digested Ad5 DNA-terminal protein complex in 293 cells and at a 10^3 -fold lower frequency on gmDBP2. This difference can readily be accounted for by the difference in transfectability of 293 versus gmDBP2.

The *Dra*I insertion mutation could not be constructed by transfections of 293 cells (Table 3). This result confirms the Vos *et al.* (1989) earlier report showing that this mutation is lethal. However, transfections of gmDBP2 followed by plaque formation on gmDBP6 showed that formation of mutant virus was only fourfold lower than that for wt virus. Several of the resulting plaques were isolated and grown to produce virus stocks. Restriction enzyme analysis of Hirt DNA from these viral isolates revealed that all of the isolates contained a *Cla*I insertion at the appropriate location in the DBP gene. The resulting mutant virus was named H5in804. Therefore, by utilizing two gmDBP cell lines, gmDBP2 for transfection and gmDBP6 for plaque isolation, nonviable DBP mutant viruses can be efficiently constructed.

H5in804 characterization

The lethality of the *Dra*I insertion mutation was established by determining the growth and plaquing efficiency of H5in804 on HeLa versus gmDBP6 cells. Although the H5in804 mutation was not completely lethal, it greatly diminished the virus's ability to replicate and form plaques on HeLa cells (Table 4). Growth was partially complemented on gmDBP6 cells, and these cells supported H5in804 plaque formation 60-fold better than HeLa cells. The kinetics of H5in804 and wt Ad5 growth on HeLa cells are compared in Fig. 4. The start of virus production in H5in804-infected cells was delayed. By 24 hr p.i. wt Ad5 had produced nearly 10^3 PFU per cell but H5in804 was not yet out of

TABLE 3

VIRUS CONSTRUCTION

Transfection condition	293 ^a		gmDBP2 ^a		gmDBP6 ^a	
	HeLa ^b	gmDBP6 ^b	HeLa	gmDBP6	HeLa	gmDBP6
1 μ g Ad5 DNA complex	1.2×10^8 ^c	1.4×10^8	6×10^3	6×10^3	0	0
1 μ g digested complex (DC)	0	0	0	0	0	0
10 μ g digested vector	0	0	0	0	0	0
DC + wt vector	2×10^5	2×10^5	3×10^2	4×10^2	0	0
DC + <i>Dral</i> insertion vector	0	0	0	1×10^2	0	0

^a Transfections were performed on the above three cell lines.

^b Three days post transfection monolayers were harvested and the level of virus produced from each condition quantitated by plaque formation on HeLa and gmDBP6 cell monolayers.

^c Amount of virus represents the total produced from one 60 mm dish of (approximately 3.5×10^6) transfected cells.

eclipse. By 36 hr p.i. wt Ad5 virus production had plateaued at 3×10^3 PFU per cell while virus production was just starting in H5in804 infections. The production of H5in804 continued to increase until 72 hr p.i., when it reached a plateau 36-fold lower than that of wt Ad5.

The reduction in virus production correlated with a reduction in viral DNA accumulation (Fig. 5A) and synthesis (Fig. 5B). The difference between H5in804 and wt Ad5 viral DNA accumulation and synthesis was most dramatic at early times. For example, at 20 hr p.i. DNA accumulation and synthesis of H5in804 was 50- to 100-fold reduced compared to that of Ad5. Peak DNA synthesis occurred at approximately 30 hr p.i. for Ad5 but had not yet been reached by 60 hr p.i. for H5in804. After 60 hr p.i. the level of DNA synthesis was difficult to quantitate for both Ad5 and H5in804 due to significant cytopathic effects, but the level of H5in804 DNA synthesis appeared to continue to in-

crease. At their respective times for maximum DNA replication, H5in804 was approximately 12-fold lower than Ad5.

DBP is one of three viral proteins required for viral DNA synthesis and is required in stoichiometric amounts. A reduced amount of DBP is therefore likely to reduce DNA replication. Therefore, the synthesis and accumulation of H5in804 DBP was determined. Synthesis (Fig. 6A) and accumulation (data not shown) of H5in804 DBP was similar to wt Ad5 under conditions where the DNA template was held constant by inhibiting DNA replication with hydroxyurea. The mobility difference observed in Fig. 6A for H5in804 corresponds to the expected increase in molecular weight

TABLE 4

GROWTH AND PLAQUING EFFICIENCY OF H5in804 ON gmDBP6 AND HeLa CELLS

Virus	Virus production (PFU/cell) ^a		Plaquing efficiency ^b gmDBP6/HeLa
	gmDBP6	HeLa	
Ad5	3000	2000	1.7
H5in804	280	29	60.0

^a Virus yield was determined by infecting the cell lines with a moi of 10 and analyzing the amount of infectious virus produced at 72 hpi by titring on gmDBP6.

^b Plaquing efficiency is the ratio of the number of plaques produced on gmDBP6 versus the number produced on HeLa cells. The values represent the average of titring 5 different viral stocks of each virus.

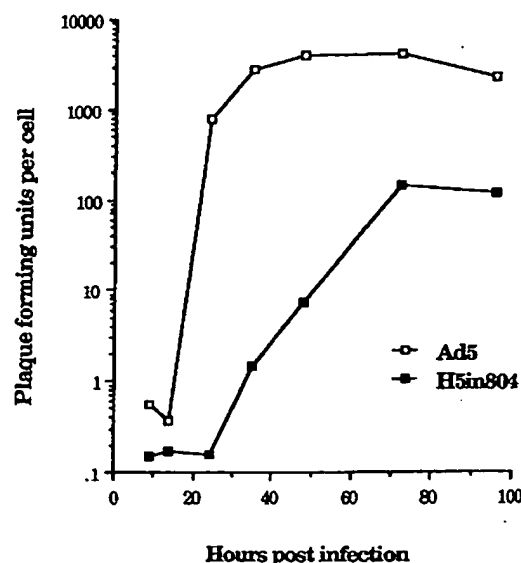


FIG. 4. Kinetics of H5in804 and wt Ad5 virus production in HeLa cells. Infected cells were harvested at the various times described and the amount of virus determined by titration of the cell lysates on gmDBP6 monolayers.

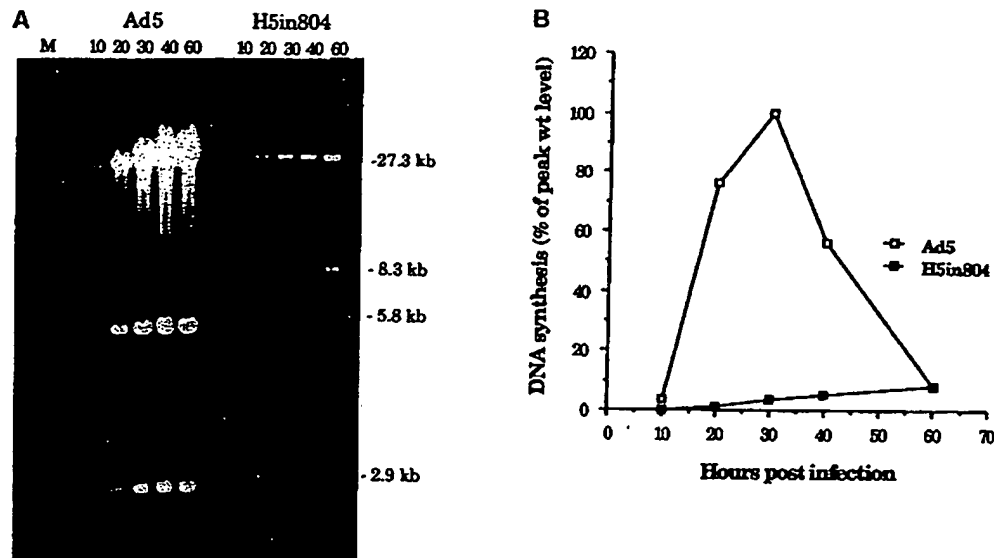


Fig. 5. Viral DNA replication in H5in804- and Ad5-infected HeLa cells. The kinetics of viral DNA accumulation (A) and synthesis (B) were compared by harvesting low-molecular-weight DNA from infected cells at the indicated time postinfection and digesting with *EcoRI* an equal fraction of each DNA preparation before electrophoresis on a 0.8% agarose gel. DNA accumulation was determined by staining the gel with ethidium bromide and visualizing. To determine the amount of DNA synthesis, monolayers were labeled for 1 hr prior to harvesting with [3 H]thymidine. The labeled, digested DNAs were transferred to nitrocellulose and detected by fluorography. The level of DNA synthesis was quantitated by densitometry, and the results were represented graphically as the percentage of peak wt Ad5 synthesis.

by the addition of 22 additional amino acids. In the absence of DNA replication inhibitors the level of H5in804 DBP was reduced compared to that of Ad5 DBP as expected. Equivalent synthesis and accumula-

tion of H5in804 DBP, in the presence of DNA replication inhibitors, suggested that the stability of the mutant protein was not affected by the mutation. This was confirmed by pulse-chase analysis which showed that H5in804 DBP was as stable as wt Ad5 DBP (Fig. 6B).

DBP is a nuclear protein which requires two nuclear localization sequences for efficient nuclear targeting (Mprin *et al.*, 1989). Although the H5in804 DBP was nuclear; in contrast to wt DBP it did not form large globular nuclear structures (data not shown). The H5in804 protein remained in a diffuse nuclear staining pattern and in small punctate structures. This is consistent with an alteration in the level of viral DNA synthesis since Voelkerding and Klessig (1986) have shown that when viral DNA replication is inhibited by drug treatment or with a temperature-sensitive mutation in the viral-encoded DNA polymerase, wt DBP exhibits a similar diffuse and small punctate pattern of staining. Therefore, the deficit in H5in804 viral DNA synthesis was not due to an alteration in level of the H5in804 DBP or to the inability of the mutant protein to reach the nucleus of the infected cell.

Equivalent levels of DBP synthesis in the absence of viral DNA replication suggested that the steady-state levels of H5in804 and Ad5 E2A (DBP) mRNA were similar. This was confirmed by Northern blot analysis (data not shown). This mRNA analysis also showed that expression of several other early regions (E1A, E1B, and E4) was also normal in H5in804-infected cells (data not

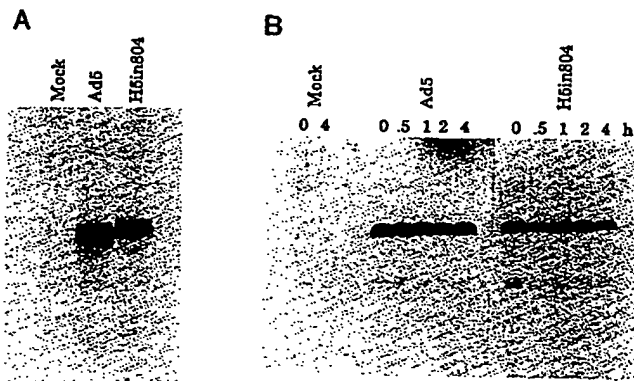


Fig. 6. Synthesis and stability of H5in804 DBP. (A) HeLa cells were infected with Ad5 and H5in804 in the presence of hydroxyurea and at 20 hr postinfection labeled with [35 S]methionine for 1 hr. DBP was immunoprecipitated, fractionated by SDS-PAGE (10% polyacrylamide gel), and autoradiographed. (B) The stability of DBP was determined by immunoprecipitating equal fractions of infected cell lysates after a 5-min [35 S]methionine pulse labeling at 20 hr postinfection in the absence of hydroxyurea, followed by a chase period as indicated with 2000-fold molar excess of unlabeled methionine. Five-fold more cell lysate was immunoprecipitated in H5in804-infected cell lysates to partially compensate for the reduction in H5in804 DBP synthesis when template levels were not kept constant by addition of hydroxyurea.

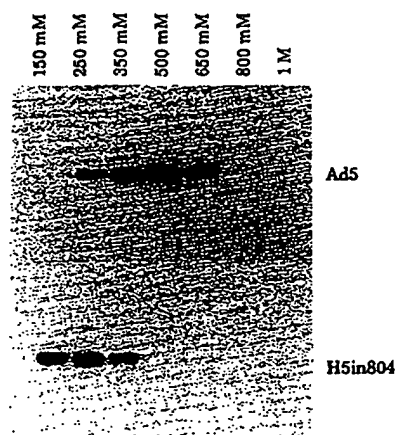


FIG. 7. Single-stranded DNA binding ability of H5in804 DBP. Infected cell extracts were ammonium sulfate precipitated (Cleghon and Klessig, 1986) to enrich for DBP in the extracts. Extracts containing approximately 1 μ g of DBP were bound to ssDNA cellulose in buffer AB plus 100 mM NaCl and eluted with increasing NaCl concentrations. Equal amounts of each fraction were separated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose, and immunoblotted for DBP.

shown). These results suggest that the reduction in DNA synthesis in H5in804 is not due to an alteration in the expression of other viral early genes and that the mutation in H5in804 does not affect DBP's complex role in early gene expression.

Binding to ssDNA is essential for DBP to perform its roles in DNA replication. To test H5in804 DBP's ability to bind to ssDNA the protein was bound to ssDNA cellulose and eluted with increasing NaCl concentrations (Fig. 7). Approximately 90–100% of wt Ad5 DBP bound to ssDNA cellulose at 100 mM NaCl and eluted between 250 and 650 mM NaCl, with a peak of elution at 500 mM NaCl. In contrast, only 60% of the H5in804 DBP bound to ssDNA cellulose at 100 mM NaCl. Moreover, the bound H5in804 DBP eluted between 150 and 350 mM NaCl. A similar binding and elution pattern was seen if the H5in804 protein was first bound at 20 mM NaCl (data not shown). Thus, the H5in804 DBP is altered in its ability to interact with ssDNA. This difference in binding probably accounts for the virus's altered level of DNA synthesis.

DISCUSSION

Transformation of HeLa cells with pMSG-DBP-EN yielded 37 independent *gpt*⁺ transformants, 31 of which expressed DBP after dexamethasone induction. The percentage of *gpt*⁺ cell lines which inducibly expressed DBP was 10-fold higher in this transformation than when cells were transformed with pMSG-DBP (Klessig *et al.*, 1984a). This dramatic increase was due to at least two factors. The first was an increase in the

frequency of integration into the host genome of an intact copy of the DBP gene. Previously with pMSG-DBP only 21% of the *gpt*⁺ cell lines contained intact copies of the DBP gene (Klessig *et al.*, 1984a), whereas with pMSG-DBP-EN a minimum of 84% of the *gpt*⁺ cell lines contained the intact DBP gene. Second, the presence of the enhancer facilitated expression of DBP. With the enhancerless vector only 42% of those lines which contained an intact DBP gene were able to express it (Klessig *et al.*, 1984a), while with the enhancer at least 84% (and perhaps all) of the cells containing the intact gene expressed it.

The new gmDBP lines were constructed in order to increase the amount of DBP expression with the expectation that this would allow plaque formation by nonviable DBP deletion mutants. When these new DBP-expressing cell lines were compared to the well-characterized gmDBP2 for accumulation of DBP, 12 of the 31 new gmDBP cell lines accumulated DBP at or above the levels seen in gmDBP2 and formed adequate monolayers. These 12 were screened for their ability to support virus plaque formation. Interestingly, none of the lines when grown on *gpt*⁺ selection supported either mutant or Ad5 plaque formation even though they sustain growth of DBP mutants. Why the selection conditions disrupted the monolayer's ability to support adenovirus plaque formation is unclear. In the absence of *gpt*⁺ selection, two-thirds (eight) allowed plaque formation by wt Ad5 and half of these (four) efficiently formed plaques with the nonviable H5dl802 deletion mutant. Except for line 22h2, there was no significant morphology difference between the two classes of cell monolayers (all cells were similar to the parental HeLa cells), nor did the higher levels of DBP expression always correlate with the ability to allow plaque formation. Moreover, while one of the H5dl802-plaquing lines (gmDBP6) allowed wt Ad5 to form plaques that appeared earlier and were more distinct, the other supporting lines showed no differences from HeLa for wt Ad5 plaque formation. Thus the success in obtaining lines that support plaque formation by DBP-nonviable mutants was probably due to the increased efficiency of producing lines that expressed the integrated DBP gene at moderately high levels. This large number of DBP-expressing lines facilitated the search for those which supported plaque formation. It appears that plaque formation depends not only on DBP levels but on an as yet undefined factor.

The true test of their utility was the use of these new gmDBP lines in construction of lethal DBP mutant viruses. Because of its plaquing properties gmDBP6 was chosen for this test. Unfortunately, we found that gmDBP6 is rather refractory to DNA transfection, thus it could not be used for the initial transfection step.

gmDBP8 also transfected poorly, suggesting that this property may be a general feature of these new lines. Nevertheless, using a combination of gmDBP2 (for transfection) and gmDBP6 (for plaquing) a new lethal DBP mutant (H5in804) was constructed. The efficiency of its construction was only slightly lower (three- to fourfold) than reconstruction of a wt virus. However, although gmDBP6 allowed for efficient plaque formation by H5in804 (60X higher than that allowed by HeLa), the mutants growth in this line was only partially complemented (Table 4). gmDBP6 accumulates 20% of the peak level of DBP seen in Ad5-infected cells. The lack of full complementation may be due to this lower level of DBP accumulation and/or improper timing (or levels) of DBP expression relative to other viral genes during the infectious cycle. Another possibility is that the mutant DBP may interfere with normal wt DBP function by interacting with the gmDBP cell line encoded wt protein or with other factors that the wt protein normally interacts.

Characterization of H5in804 revealed that the reduction in virus production resulted from an alteration in the virus's ability to replicate DNA. This decrease in DNA synthesis does not appear to be due to an alteration in level or nuclear location of the H5in804 DBP in infected HeLa cells. Furthermore, analysis of early mRNA suggested that the H5in804 phenotype was not due to changes in the expression of other viral early genes. However, analysis of the mutant DBP *in vitro* showed that its binding affinity for ssDNA was reduced. Since binding to ssDNA is essential for DBP to function in DNA replication, this difference most likely accounts for the reduction in DNA synthesis and hence the debilitation of the virus.

The C-t domain of DBP is responsible for ssDNA binding. Mutations in this domain affect its binding and proteolytic fragments containing most or all of this domain retain binding activity. Thus, perhaps it is not surprising that the H5in804 mutation alters ssDNA binding. However, the mutation does not directly alter or delete any amino acids contained within this domain but only extends the polypeptide 22 amino acids. While this most likely would impact the very C-t segment of the polypeptide, this effect should be minimal since a proteolytic fragment in which the last 60 amino acids are removed retain binding activity (Cleghon and Klessig, 1992). Therefore, this extension mutation most probably sterically interferes directly with the protein's ability to interact with ssDNA or may alter the folding (and hence the conformation) of the protein.

ACKNOWLEDGMENT

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A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2

(complementing cell line/*gpt* selection/mycophenolic acid)

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ABSTRACT Cell lines that produce viral gene products and that can support the growth of viral mutants lacking those products have been valuable in the genetic analysis of the transforming regions of several animal viruses. To extend the advantages of such complementing cell systems to regions of the adenoviral genome not directly involved in transformation, we have constructed a cell line that will support the growth of a defective adenoviral deletion mutant, H2d1808, that lacks most of early region 4 (E4). The right-hand terminal adenovirus 5 *Eco*RI restriction fragment, which contains all of E4, was first inserted into the vector pSV2*gpt*, and the recombinant plasmid was introduced into Vero cells by calcium phosphate precipitation. Clones containing the hybrid plasmid were selected by their resistance to mycophenolic acid. Five mycophenolic acid-resistant clones were then tested for the ability to support the growth of H2d1808. One of the five lines, W162, permits plaque formation by H2d1808 at an efficiency that is $>10^6$ -fold higher than that of the parental Vero cells and allows the production of high-titer, helper-free H2d1808 stocks. Thus, W162 cells are permissive for at least one defective E4 mutant. The line carries, as expected, an intact E4, detected by hybridization. Using an H2d1808 lysate produced on W162 cells, we have accurately mapped the 808 deletion. It extends from between *Bcl*I and *Sma*I sites at positions 91.4 and 92.0, respectively, to just beyond a *Hind*III site at position 97.2 and, therefore, falls entirely within E4. H2d1808 and W162 should be of value in determining the physiological role of E4 in adenoviral infection.

Studies that probe the functions of viral genes are frequently dependent upon the availability of viral mutants. Most of the mutants that have proved useful in studying viral gene function are conditionally defective; in animal virus systems, the majority of these are temperature sensitive (1, 2). An alternative approach to the isolation of conditionally lethal mutations exploits the fact that some virally transformed cells will support the growth of mutants with defects in transforming regions. For example, polyoma virus-transformed mouse cells support the growth of *hr-t* mutants which carry lesions in the polyoma early region (3); COS cells, a line of simian virus 40 (SV40)-transformed monkey cells, support the growth of SV40 early region mutants (4); and 293 cells, a line of human cells transformed by sheared adenoviral DNA (5), support the growth of mutants of adenoviral early region 1 (6-8). In each of these cases, mutant viruses can be propagated efficiently on the transformed cell line, and their phenotypes subsequently can be analyzed in normal, nonpermissive cell types. The value of a complementing cell line in the isolation and propagation of viral mutants is probably best illustrated by the last example; the examination of a wide variety of mutants of adenoviral early region 1 (E1) has provided a detailed picture of the functions of that region (6-13).

In the examples cited above, the expression of integrated viral DNA is presumably responsible both for the cells' transformed phenotype and for their ability to complement the defective mutants. However, many segments of viral DNA do not transform cells, and there is no direct selection for cells that contain such DNA and that might support the growth of mutants in those regions of a viral genome. In an effort to extend the complementing cell approach to segments of viral DNA that do not transform cells and to make possible the analysis of mutants of adenoviral early region 4 (E4), we have used the *Escherichia coli gpt*-based selective system of Mulligan and Berg (14, 15) to introduce E4 DNA into cells that are permissive for human adenoviruses. E4 lies at the right end of the adenoviral genome, and although it is required for viral growth (see below), its role in the viral life cycle is not known. E4 is genetically ill-characterized, and so a cell line that would complement E4 mutants and simplify their isolation and analysis would be useful. One of the lines that we have obtained supports the growth of a defective adenoviral mutant, H2d1808 (16), which lacks most of E4. This paper describes the isolation of this line, its partial characterization, and the accurate mapping of the 808 deletion.

MATERIALS AND METHODS

Cells and Viruses. Vero cells were obtained from A. M. Lewis and 293 cells from F. Graham. Vero cells were grown in monolayers in Eagle's minimal essential medium containing 10% calf serum (medium A), and 293 cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (medium B). Mycophenolic acid-resistant Vero cell derivatives were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and selective drugs as described below.

Wild-type adenovirus type 2 (Ad2) was originally from A. Lewis. H2d1808 is an Ad2 deletion mutant lacking the viral DNA between about positions 92 and 97.1 on the standard map; its isolation was described by Challberg and Ketner (16).

Transformation and Mycophenolic Acid Selection. Derivatives of the pSV2*gpt* plasmid of Mulligan and Berg (14, 15) containing adenoviral DNA (see *Results*) were introduced into Vero cells by the calcium phosphate precipitation technique (17, 18). About 5×10^5 cells were plated in a 9-cm Petri dish on the day before they were to be transformed; 15-24 hr later, the medium was removed from the dishes, and 0.5 ml of a suspension of precipitated DNA (12.5 μ g of plasmid DNA per 0.5-ml aliquot) was added to each dish. After 20 min at room temperature, the plates were filled with 9 ml of medium A and transferred to a 37°C incubator. Four hours later this medium was replaced with selective medium: Dulbecco's modified Eagle's medium containing calf serum (10%), mycophenolic acid (25 μ g/ml), xanthine (250 μ g/ml), hypoxanthine (15 μ g/ml),

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Abbreviations: Ad2, adenovirus type 2; Ad5, adenovirus type 5; E1 and E2, early regions 1 and 2 of adenoviral genome; SV40, simian virus 40.

amethopterin (2 $\mu\text{g}/\text{ml}$), and thymidine (10 $\mu\text{g}/\text{ml}$) (16). Mycophenolic acid was the generous gift of the Eli Lilly Research Laboratories. Mycophenolic acid-resistant colonies first became visible 7–9 days later and were picked after 14–20 days by using cloning cylinders cut from plastic Eppendorf centrifuge tubes. The transformants were grown up and are maintained in the selective medium. It is at present unclear whether continued selection is necessary if W162 cells are to retain their biological activity over long periods, although a brief period without selection does not affect complementing ability (see below).

Preparation of W162 Monolayers for Plaque Assays. To conserve mycophenolic acid, plaque assays on W162 monolayers were performed in the absence of selective drugs. When suddenly withdrawn from selective medium, however, W162 cells grew very poorly. This difficulty could be avoided by a single passage through Eagle's minimal essential medium containing hypoxanthine, xanthine, and thymidine without amethopterin and MPA (medium C). Therefore, W162 cells to be used for plaque assays were transferred first into medium C and, after 2 to 3 days, into 5-cm dishes containing Eagle's minimal essential medium. The resulting monolayers could be used without further special treatment.

Southern Transfers and Hybridization. Cellular DNAs, digested with the restriction endonucleases *EcoRI* or *HindIII*, were fractionated on 3-mm thick vertical slab gels and transferred to nitrocellulose filter sheets by the Southern procedure (19). Restriction fragments containing adenovirus type 5 (Ad5) sequences were detected by hybridization (20, 21) to adenoviral DNA labeled with ^{32}P by nick-translation (22) and subsequent autoradiography.

RESULTS

Construction of Cell Lines. The initial goal of these experiments was to introduce adenoviral early region 4 (E4) into a cell line permissive for human adenoviruses. To do so, we used the *gpt*-based selective system developed by Mulligan and Berg (14, 15). This system permits the selection of cells that take up one of a series of plasmid vectors containing the *E. coli gpt* gene linked to SV40 sequences that allow its expression in animal cells. The basis of the selection is the novel ability of such cells to utilize exogenous xanthine as a source of GMP, when *de novo* GMP synthesis is blocked by the drug mycophenolic acid: *gpt*-containing cells are resistant to mycophenolic acid in the presence of xanthine, whereas normal cells are not. Mycophenolic acid resistance is dominant, and the recipient cells need not possess any special properties. We constructed two derivatives of one of the *gpt* vectors, pSV2*gpt*. These plasmids (pE4*gpt*6 and pE4*gpt*16) both contain the Ad5 *EcoRI* B fragment inserted at the vector's single *EcoRI* cleavage site but differ in the orientation of the viral DNA segment. Ad5 *EcoRI* B covers the region 84–100 on the viral genome and contains all of E4, the fiber gene, and part of early region 3. The Ad5 *EcoRI* B fragment that we used has been modified by the addition to its right end of a synthetic *EcoRI* site and was kindly provided by K. Berkner. pE4*gpt*16 is diagrammed in Fig. 1.

The two E4-bearing plasmids were introduced into Vero cells by calcium phosphate precipitation, and mycophenolic acid-resistant clones were selected. On the average, two or three transformants were obtained from each plate exposed to plasmid DNA. A total of 13 clones were picked, 5 made with pE4*gpt*6 and 8 made with pE4*gpt*16. Four clones produced with pE4*gpt*16 (W162 through W165) and one clone produced with pE4*gpt*6 (W6B) were selected for further examination.

Assay for Complementing Activity. To determine whether any of the selected mycophenolic acid-resistant cell lines were capable of complementing an E4 defect, we tested each one for

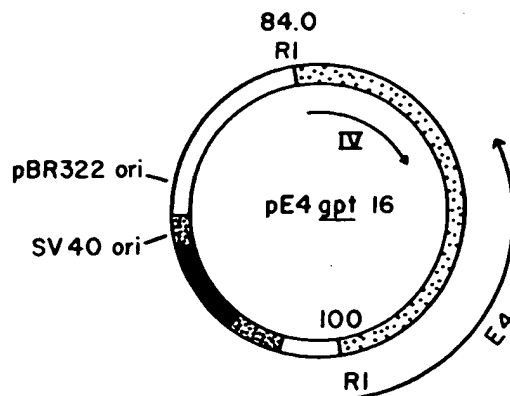


FIG. 1. Structure of pE4*gpt*16. pE4*gpt*16 consists of the Ad5 *EcoRI* B fragment (positions 84–100) inserted at the single *EcoRI* site of pSV2*gpt* (14). On the map, adenoviral sequences are indicated by light stippling, SV40 sequences by heavy stippling, and *E. coli gpt* sequences by the solid bar. The coordinates indicated are from the adenovirus physical map. Arrows indicate the direction of transcription and extent of adenoviral E4 and of the fiber gene (IV).

the ability to support growth of the defective E4 deletion mutant H2*dl*808 (16). The 808 deletion covers sequences from about 92 to about 97 map units on the Ad2 genome (see below) and, thus, is entirely within E4 (23, 24). A mixed stock containing both H2*dl*808 and its Ad5 *ts* helper, enriched for the deletion mutant by four cycles of CsCl equilibrium density gradient centrifugation, was titrated on each of the five mycophenolic acid-resistant lines listed above. The apparent titer of the stock on the five lines ranged from about 2×10^5 plaque-forming units/ml (W162, W163, W165, and W6B), to about 3×10^4 plaque-forming units/ml (W164). Five plaques produced on each of the lines W162–W165 and 10 plaques produced on W6B were picked and used to produce small lysates in cells of the same line. These lysates were then used to prepare small amounts of ^{32}P -labeled viral DNA, which were digested either with *EcoRI* or *Xba* I and analyzed by agarose gel electrophoresis. Judged by the restriction fragments produced, all of the plaques formed on four of the lines (W163–W165 and W6B) contained H2*dl*808 and its Ad5 helper, the helper alone, or Ad2/Ad5 recombinants lacking the 808 deletion. Therefore, none of these lines seems to support the growth of pure H2*dl*808. In contrast, three of the five plaques picked from lawns of W162 contained only H2*dl*808. No restriction fragments characteristic of the Ad5 helper were observed in digests of DNA from these plaques, and the Ad2 fragments affected by the 808 deletion (*EcoRI* C and *Xba* I C), were entirely replaced by the expected novel fragments. The remaining plaques contained both H2*dl*808 and Ad5. An *EcoRI* digest of DNA from descendants of one of the mutant plaques, subsequently replaques and grown up on W162, is presented in Fig. 2.

Viral Growth on W162. To confirm that H2*dl*808 is defective on normally permissive cell lines and that it forms plaques efficiently on W162, we titrated Ad2 and H2*dl*808 stocks on W162 and on the parental Vero strain. The H2*dl*808 stock used was produced in W162 cells from virus purified by three successive rounds of plaque formation on W162 monolayers. As shown in Table 1, H2*dl*808 formed plaques more than 10^5 -fold more efficiently on W162 cells than on Vero monolayers. We conclude that W162 complements a defect in H2*dl*808 that renders the mutant defective. That lesion is presumably the 808 deletion; thus, it seems certain that W162 will complement at least some defective E4 mutants.

Viral DNA in W162. Because we expected the complementing activity of W162 to be dependent upon the presence

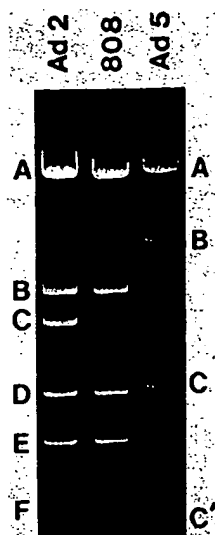


FIG. 2. *EcoRI* digest of H2dl808 DNA. DNAs obtained from purified H2dl808, Ad2, and Ad5 virions were digested with the *EcoRI* restriction endonuclease. The resulting fragments were fractionated by electrophoresis on a 1.4% agarose gel, stained, and photographed. In the digest of mutant DNA, the Ad2 C fragment is replaced by a shortened derivative (C'), which is slightly smaller than Ad2 *EcoRI* F.

of viral E4 DNA, we assayed W162 for viral DNA sequences by the Southern transfer procedure (19). W162, Vero, and 293 DNAs (10 μ g each) were digested with either the *HindIII* or *EcoRI* restriction endonucleases, fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized to 32 P-labeled Ad2 DNA. The *HindIII* digest of W162 DNA contained three bands that hybridized to viral DNA (Fig. 3). One of these comigrated with Ad5 *HindIII* F (89.5–98.2 map units), which contains most of E4. *EcoRI* digestion of W162 DNA produced one fragment containing viral sequences that had a slightly greater mobility than had Ad5 *EcoRI* B. Vero DNA contains no viral sequences, whereas 293 DNA digested with either enzyme yielded three fragments that hybridized with viral DNA. Because the fragment observed in the *EcoRI* digest of W162 DNA did not comigrate precisely with the Ad5 *EcoRI* B marker, the pE4_{gpt16} DNA present in these cells must have suffered some rearrangement during its incorporation. However, both the phenotype of the line and the presence of the *HindIII* F fragment suggest that W162 carries an intact, functional E4.

The 808 Deletion. The deletion in H2dl808 had previously been mapped by electron microscopy to coordinates 92.0–97.1 (14). To refine these measurements, we assayed DNA obtained from plaque-purified H2dl808 virions for the presence of several restriction sites near the ends of the deletion. The results of these mapping experiments (summarized also in Fig. 4) indicate that *Sma* I and *HindIII* cleavage sites at positions 92.0 and 97.2 (25), respectively, are missing from H2dl808 DNA, while *Bcl* I and *Sma* I sites at positions 91.4 and 98.4 (25), re-

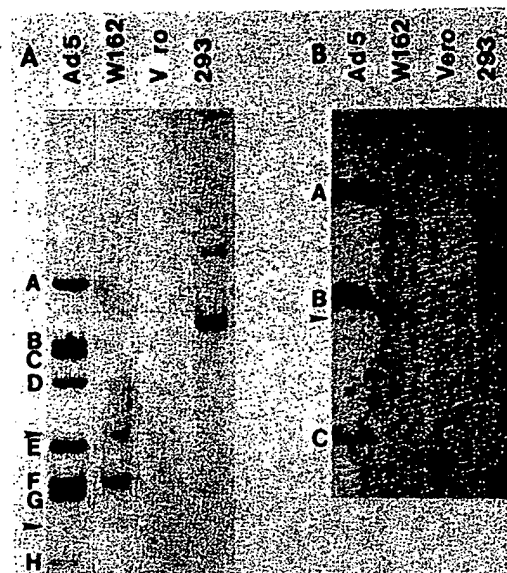


FIG. 3. Adenoviral DNA sequences in W162. W162, Vero, and 293 DNAs (10 μ g each) were digested with *HindIII* or with *EcoRI*, transferred to nitrocellulose by the Southern procedure (19), and hybridized to 32 P-labeled Ad2 DNA. (A) *HindIII* digests. The eight largest *HindIII* fragments are indicated by letters. The arrowheads mark the positions of two of the bands that contain viral sequences in the lane containing W162 DNA; the third band lies next to *HindIII* F. (B) *EcoRI* digests. The three Ad5 *EcoRI* bands are labeled, and an arrowhead marks the position of the band containing viral sequences in the digest of W162 DNA. The Ad5 standard contains viral DNA equivalent to about 10 copies per genome.

spectively, are present. Therefore, the left end point of the deletion falls in the roughly 200-base region between positions 91.4 and 92.0, and the right end point falls between positions 97.2 and 98.4. The size of the 808 deletion, measured by electron microscopy and estimated from the mobility of the novel restriction fragments produced in H2dl808 DNA by the deletion (Fig. 2) is just over 5%. It is likely, therefore, that the right end point lies quite close to the *HindIII* site at position 97.2 as shown in Fig. 4.

The 808 deletion, which does not cover the *Bcl* I site at position 91.4, cannot be any closer to the presumed polyadenylation site for fiber mRNA (position 91.1; refs. 25 and 26) than about 100 bases. It is likely, therefore, that fiber mRNA is not directly affected by the 808 deletion. This is of particular interest in light of the observation that, even in W162 cells, H2dl808 substantially underproduced fiber protein (data not shown). Therefore, the 808 deletion may define a downstream site, outside of the sequences incorporated into stable mRNA,

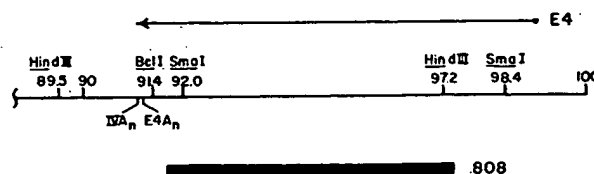


FIG. 4. Map of the H2dl808 deletion. The extent of the H2dl808 deletion, deduced from restriction digests and electron microscopy, is diagrammed. The end points of the deletion (black bar) fall between a *Bcl* I site at position 91.4 and a *Sma* I site at position 92.0 on the left and between a *HindIII* site at position 97.2 and a *Sma* I site at position 98.4 on the right. The positions of the presumed polyadenylation signals for fiber (IVA_n) and E4 (E4 A_n) RNAs and the approximate extent of E4 transcription (arrow) are indicated (23, 24).

Table 1. Titration of Ad2 and H2dl808 on W162 and Vero cells

	Ad2	H2dl808
W162	4.0×10^7	7×10^7
Vero	1.5×10^7	$<1 \times 10^{1*}$

Ad2 and H2dl808 lysates were titrated on W162 and Vero cells. The titers of the two stocks on these cell lines appear above, expressed as plaque-forming units per ml.

* No plaques appeared on either of two dishes inoculated with 1.0 ml of the H2dl808 stock diluted 1:10.

that is necessary for efficient expression of the fiber gene. More thorough analysis of H2dl808 will presumably shed light on this possibility and on the nature of the mutant's biochemical defect.

DISCUSSION

The genetic analysis of the transforming regions of several animal viruses has been facilitated by the fact that some transformed cell lines support the growth of otherwise defective viral mutants with lesions in those regions. Such transformed cell lines contain and express segments of viral DNA (3–5) and presumably are capable of supplying the essential products of those DNA segments to viral mutants that cannot produce them. The experiments described here were undertaken to extend the benefits of such complementing cell systems to a viral DNA segment not directly involved in transformation; early region 4 of human adenoviruses. In these experiments, a segment of adenoviral DNA containing early region 4 sequences derived from Ad5 was introduced into Vero cells as part of a plasmid containing the Ad5 *EcoRI* B fragment and the dominant, selectable *E. coli* gene *gpt* (14). Several of the resulting cell lines were then screened for biological activity and one, W162, was found to support the growth of the defective E4 deletion mutant H2dl808 (16). Thus, it is possible to construct complementing cell lines for at least some segments of viral DNA that do not transform cells, and a line that should be useful in the analysis of E4 was obtained. Recently, Shiroki *et al.* (27) and Babiss *et al.* (28) reported the use of the *gpt* selective system to construct KB cell derivatives containing adenoviral E1 sequences. Like 293 cells, some of these lines support the growth of E1 mutants.

Vero cells, which are of monkey origin but permissive for human adenoviruses, were chosen for these experiments rather than a human cell line partly for technical reasons: Vero cells grow well, form durable monolayers, and were easily transformed to mycophenolic acid resistance. Further, in Vero derivatives, resident E4 sequences should remain silent because Vero cells do not contain the adenoviral E1 sequences required for efficient E4 expression (9, 10). Thus, even if E4 expression is lethal, there ought to be no selection against E4-containing Vero transformants; as there might be against similar derivatives of, for example, 293 cells. We presumed that in E4-bearing Vero derivatives, infecting adenovirus would activate the resident E4 DNA by providing E1 products.

Five mycophenolic acid-resistant lines, all of which presumably carry the *gpt* gene and attached E4 DNA, were originally chosen for close examination. Of these, only one seems to complement the E4 mutant that we have used to test biological activity. The reason for the inactivity of the other four lines is not known. All of the lines examined, including W162, grow well and form long-lived monolayers.

E4 is one of the few segments of the adenoviral genome for which no function in the viral life cycle is known. This is due in part to the lack of E4 mutants: no conditionally defective mutants are available, and H2dl808, which until now has been propagated in the presence of a helper virus, carries a deletion too small to make its physical purification practical. One deletion mutant lacking E4 sequences (H2dl807; ref. 14) has been characterized, but the interpretation of its phenotype is complicated by the fact that it is missing a substantial amount of DNA outside of E4. The difficulties encountered in the genetic analysis of E4 should be considerably reduced by W162, which will make the analysis of H2dl808 possible immediately and should permit the eventual isolation of new E4 mutants.

Using lysates produced on W162 cells, we have begun the characterization of H2dl808, which lacks viral sequences from between positions 91.4 and 92.0 to just beyond position 97.2.

This deletion falls entirely within E4 (ref. 24; see Fig. 4) and would disrupt all but the most promoter-proximal of the hypothetical protein-coding regions in E4 proposed on the basis of sequence data (25, 26). We are not yet certain of the level at which the growth of H2dl808 is blocked in nonpermissive cells. It is of interest that even in W162 cells, H2dl808 synthesizes little fiber protein. Because fiber mRNA ought not to be directly affected by the deletion, the missing DNA may contain a novel genetic element required for efficient expression of the fiber gene.

The W162 cell line should soon shed light on the functions of adenoviral early region 4. The method used in the construction of the line also should be of general utility in producing similar complementing cell lines for other regions of interest in animal virus genomes.

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Isolation and Analysis of Adenovirus Type 5 Mutants Containing Deletions in the Gene Encoding the DNA-Binding Protein

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A genetic system is described which allows the isolation and propagation of adenovirus mutants containing lesions in early region 2A (E2A), the gene encoding the multifunctional adenovirus DNA-binding protein (DBP). A cloned E2A gene was first mutagenized *in vitro* and then was introduced into the viral genome by *in vivo* recombination. The E2A mutants were propagated by growth in human cell lines which express an integrated copy of the DBP gene under the control of a dexamethasone-inducible promoter (D. F. Klessig, D. E. Brough, and V. Cleghon, *Mol. Cell. Biol.* 4:1354-1362, 1984). The protocol was used to construct five adenovirus mutants, Ad5d1801 through Ad5d1805, which contained deletions in E2A. One of the mutants, Ad5d1802, made no detectable DBP and thus represents the first DBP-negative adenovirus mutant, while the four other mutants made truncated DBP-related polypeptides. All five mutants were completely defective for growth and plaque formation on HeLa cell monolayers. Furthermore, the two mutants which were tested, Ad5d1801 and Ad5d1802, did not replicate their DNA in HeLa cells. The mutant Ad5d1804 encoded a truncated DBP-related protein which contained an entire amino-terminal domain derived from the host range mutant Ad5hr404, a variant of Ad5 which multiplies efficiently in monkey cells. While results of a previous study suggest that the amino-terminal domain of DBP could act independently of the carboxyl-terminal domain to enhance late gene expression in monkey cells, the Ad5d1804 polypeptide failed to relieve the block to late viral protein synthesis in monkey cells. The mutant Ad5d1802 was used to study the role of DBP in the regulation of early adenovirus gene expression in infected HeLa cells. These experiments show that E2A mRNA levels are consistently reduced approximately fivefold in Ad5d1802-infected cells, suggesting either a role for DBP in the expression of its own gene or a *cis*-acting defect caused by the E2A deletion. DBP does not appear to play a significant role in the regulation of adenovirus early regions 1A, 1B, 3, or 4 mRNA levels in infected HeLa cell monolayers since wild-type Ad5- and Ad5d1802-infected cells showed very little difference in the patterns of expression of these genes.

The early region 2A gene (E2A) of human adenovirus types 2 and 5 (Ad2 and Ad5, respectively) encodes a 72-kilodalton (kDa) protein which possesses single-stranded DNA binding activity (34, 46). This polypeptide, commonly called the adenovirus DNA-binding protein (DBP), is thought to contain two physical (25, 43) as well as functional (4, 31, 40) domains. A large number of functions have been ascribed to DBP. The best characterized of these is the role of the protein in viral DNA replication. DBP is required for the strand elongation reaction of viral DNA synthesis (12, 16) and may play a role in the initiation reaction as well (17, 37, 47). This polypeptide has also been implicated in the regulation of adenovirus early gene expression. Results of studies involving the microinjection of purified adenovirus genes or mRNAs into cells have suggested that DBP may stimulate both early region 1B (E1B) (42) and early region 4 (E4) (41) gene expression. DBP has also been proposed to have a negative influence on early gene expression at intermediate and late times after infection. Evidence for this comes primarily from studies with the temperature-sensitive (*ts*) E2A mutant Ad5ts125. This mutant, unlike wild-type (WT) adenovirus, fails to turn down early gene expression as viral infection proceeds and thus accumulates higher

amounts of early mRNAs than WT virus (10, 11). The negative regulation mediated by DBP is thought to affect both the cytoplasmic stabilities of early region 1A (E1A) and E1B mRNAs (7) as well as the rate of transcription of E4 (38). More recently, repression of E4 transcription by DBP has been demonstrated *in vitro* (23). A further function for DBP in late viral gene expression has been revealed by studying abortive infections of human adenovirus in monkey cells. WT virus fails to grow in monkey cells because of a complex block to viral late gene expression (8, 14, 26). This block involves a reduced rate of transcription across the late viral gene block (J. M. Johnston, K. P. Anderson, and D. F. Klessig, submitted for publication), altered splicing of the mRNA encoding the fiber polypeptide (3, 28), and poor *in vivo* translation of the fiber mRNA (2). Adenovirus host range (*hr*) mutants which overcome these blocks and grow productively in monkey cells contain mutations in the amino-terminal segment of the E2A gene which cause a substitution of tyrosine for histidine at amino acid 130 of DBP (1, 9, 32). DBP also can affect the efficiency with which adenovirus transforms rat cells, as Ad5ts125 transforms cells at a three- to eightfold enhanced frequency compared with WT virus (19, 49). Finally, results of a recent study have suggested that DBP may play a role in the assembly of virus particles (39).

Although the small number of *ts* and *hr* mutants isolated to date have been invaluable in ascribing functions to DBP, it is apparent that a larger collection of mutants will be needed to understand this multifunctional protein in more detail. How-

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ever, since several of the activities of DBP are essential for virus growth, many potential E2A mutants are expected to have a nonconditionally lethal phenotype and thus cannot be isolated. To circumvent this problem, our laboratory has engineered several human cell lines, designated gmDBP cells, which contain and express integrated copies of the E2A gene (27). Since it was suspected that expression of DBP is toxic to cells (30), the E2A gene in these cell lines was under the control of a glucocorticoid hormone-inducible promoter. In the presence of dexamethasone, a synthetic glucocorticoid hormone, gmDBP1 and gmDBP2 cells induce DBP synthesis 50- to 200-fold to a level that is 5 to 20% of the peak synthesis level seen in adenovirus-infected HeLa cells (27).

The gmDBP cell lines therefore can theoretically serve as a permissive system for the isolation and propagation of E2A mutants. A similar strategy was used to isolate a wide variety of mutants mapping in E1A and E1B. These mutants are efficiently complemented by the human 293 cell line which expresses an integrated copy of E1A and E1B (20). DBP differs from the gene products encoded by E1A and E1B, however, in that it is probably required in very large amounts (up to 10^7 molecules per cell) during a viral infection. Although the gmDBP cell lines complement the growth of E2A *ts* mutants (27), the *ts* mutations probably inactivate only a subset of the functions of DBP (40). Thus it was unclear whether these cell lines would allow the isolation of DBP-negative adenovirus mutants.

In this report we describe a genetic system which has allowed the construction of five E2A deletion mutants. All of the mutants can be propagated in the gmDBP cell lines in the presence of dexamethasone. Since at least one of the mutants makes no functional DBP, the gmDBP cell lines should serve as a useful system to isolate E2A mutants of any desired genotype. The phenotypes of the five deletion mutants have shown, as expected, that DBP is an essential adenovirus protein required for viral DNA replication. Experiments are presented which utilize the E2A deletion mutants to investigate the role of DBP in the regulation of early and late viral gene expression.

MATERIALS AND METHODS

Cells, viruses, and infections. The human 293 and HeLa cell lines were obtained from J. F. Williams. CV₁ and CV₂ cells, established lines of African green monkey kidney cells, were obtained from J. Mertz and P. Tegtmeyer, respectively. Isolation of the E2A-complementing gmDBP1 and gmDBP2 cell lines has been described recently (27). The gmDBP2a cell line was isolated as a subclone of gmDBP2 cells. Monolayer cultures of 293, HeLa, and gmDBP cells were cultivated in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% calf serum (Irvine Scientific), 100 μ g of streptomycin per ml, 100 μ g of penicillin per ml, and 292 μ g of glutamine per ml. For CV₁ and CV₂ cultivation, 5% fetal calf serum (Flow Laboratories) was used instead of 10% calf serum.

Ad2 and Ad5 were originally obtained from U. Pettersson and J. F. Williams, respectively. Ad2hr400 and Ad5hr404 have been isolated by Klessig and Grodzicker (29). All of the viruses identified above were propagated in suspension cultures of HeLa cells. Ad5ts125 and Ad5d1434 were obtained from J. F. Williams and D. Solnick, respectively. Ad5ts1+2 was isolated after ligating the *Bam*HI A restriction fragment (coordinates 0 to 59.5) derived from Ad5ts2 (48) to the *Bam*HI B restriction fragment (coordinates 59.5 to 100)

derived from Ad5ts1 (48). The ligated DNA was used to transfect 293 cells at 33°C. Plaques were picked and screened for the inability to complement either of the parental viruses at the nonpermissive temperature. A positive isolate was plaque purified and designated Ad5ts1+2. Ad5ts125 and Ad5ts1+2 were propagated in monolayers of HeLa cells at 33°C, and Ad5d1434 was propagated in 293 cells at 37°C. Simian virus 40 (SV40) strain 776 was obtained from J. Sambrook, and stocks were prepared in CV₁ cells. Plaque assays for all virus except Ad5d1801 through Ad5d1805 were performed as described previously (22). The isolation, propagation, and assay of the E2A deletion mutants Ad5d1801 through Ad5d1805 are described in detail below.

All virus infections were done on confluent cell monolayers. Virus inocula were adsorbed at 37°C for 75 min in phosphate-buffered saline. The cell monolayers were washed, overlaid with media, and incubated at 37°C unless otherwise indicated.

Construction of plasmids containing E2A deletions. The first set of E2A deletions (d1801 and d1802) was constructed in the plasmid pAd2SA (obtained from U. Pettersson) which contains the Ad2 *Sma*I A fragment (adenovirus coordinates 56.9 to 75.8) cloned in the *Pst*I site of pBR322. This plasmid contains a unique *Xho*I restriction site at coordinate 65.9 in the amino-terminal portion of the DBP-coding region. It was necessary to use an Ad2 rather than an Ad5 clone because of Ad5 E2A gene does not contain this *Xho*I site. One microgram of plasmid DNA was first linearized at its unique *Xho*I site (coordinate 65.9 in the adenovirus insert) and then digested in a volume of 50 μ l with 1 U of *Bal* 31 nuclease (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) for 20 s at 30°C. The DNA was extracted with phenol and chloroform-isoamyl alcohol, religated with T4 DNA ligase, and used to transform *Escherichia coli*. Plasmid DNA isolated from individual transformants (24) was screened by restriction enzyme digestion to identify those plasmids which contained deletions entirely within the DBP-coding region. Two such plasmids were obtained and designated pAd2SA-801 and pAd2SA-802.

The second set of E2A deletions (d1803 through d1805) was constructed with the plasmid pAd5hrKE, which was derived by cloning the Ad5hr404 *Kpn*I E fragment (coordinates 61.3 to 71.4) into the *Pvu*II site of pBR322 with *Kpn*I linkers (New England Biolabs, Inc., Beverly, Mass.). Ad5hr404 is an *hr* mutant of Ad5 which has acquired the ability to grow productively in monkey cells because of a point mutation in the amino-terminal segment of the E2A gene (29, 32). The pAd5hrKE plasmid contains three *Sst*II restriction enzyme sites, all of which reside in the DBP gene at coordinates 62.2, 64.4, and 65.7. One hundred micrograms of pAd5hrKE DNA was digested to completion with *Sst*II. The three resulting fragments were separated by electrophoresis in a 1.6% agarose gel and purified by electrophoresis into dialysis bags and passage over DEAE-Sephacel (35). Both purified insert fragments (the fragments at coordinates 62.2 to 64.4 and 64.4 to 65.7) were individually ligated into the vector backbone, and the DNA was used to transform *E. coli*. DNA from transformants was screened by restriction enzyme analysis to identify clones which contained single inserts in the proper orientation or no insert. Clones containing the deletions from coordinates 62.2 to 65.7, 64.4 to 65.7, and 62.2 to 64.4 were designated pAd5hrKE-803, pAd5hrKE-804, and pAd5hrKE-805, respectively.

The five E2A deletions were next introduced into the pAd5HA plasmid, which consists of the *Hind*III A fragment

of Ad5 cloned into pBR322. To accomplish this, the deletion plasmids were digested with *Kpn*I, and the insert fragments (coordinates 61.3 to 71.4) were purified as described above. These were then cloned in the proper orientation into the purified pAd5HA vector backbone (pAd5HA minus its own insert) to give rise to the plasmids pAd5d1801 through pAd5d1805.

The extents of the deletions in pAd5d1801 and pAd5d1802 were analyzed by DNA sequence analysis (36). The pAd5d1801 plasmid contains a 313-base-pair (bp) deletion which fuses bp 22 of the DBP-coding region (the A of the initiating ATG is defined as bp 1) to bp 336. The pAd5d1802 plasmid contains a 242-bp deletion which fuses bp 64 to bp 307. The plasmids pAd5d1803 through pAd5d1805 contain precise deletions of one or both *Ssr*II fragments as the new joints were digestible with *Ssr*II and therefore are deleted for 1272, 802, and 470 bp, respectively. The deletions in pAd5d1803 through pAd5d1805 fuse bp 216 to bp 1489, bp 686 to bp 1489, and bp 216 to bp 687, respectively.

Construction, propagation, and plaque assay of the adenovirus E2A deletion mutants. Genomic DNA-terminal protein complex was prepared from Ad5ts1+2 by the method of Chinnadurai (13), dialyzed extensively in 10 mM Tris (pH 7.8)–1 mM EDTA–1 mM β -mercaptoethanol, and stored at 4°C. The DNA-protein complex (0.5 to 1 μ g) was mixed with 6 adenovirus molar equivalents of *Hind*III-digested pAd5d1801 through pAd5d1805 and transfected by the calcium phosphate procedure (21, 29) into 293 or dexamethasone-treated gmDBP1 cells. The transfected cells were incubated at 33°C for 4 days. Progeny virus were released from the cells by freeze-thawing and sonication. Four five-fold serial dilutions of each lysate were made in phosphate-buffered saline, and 0.5 ml of each dilution was used to infect monolayers (60 mm) of gmDBP1 or gmDBP2 cells. The infections were incubated for 3 to 6 days at 40°C in media containing 0.6 μ M dexamethasone. The progeny of these infections were used to infect fresh monolayers of gmDBP1 cells, and this process was continued until the cultures exhibited cytopathic effects (CPE). Large stocks of the deletion mutants were made by infecting gmDBP2 or gmDBP2a monolayers (60 to 100 100-mm plates) in the presence of 0.6 μ M dexamethasone. The infected cells were incubated at 37°C and harvested at 68 to 80 h postinfection (p.i.).

The infectious titer of the E2A deletion mutant stocks was determined by plaque assay on HeLa cells using Ad5d1434 as a helper virus. Plaque assays were performed as usual, except the HeLa cell monolayers were simultaneously infected with Ad5d1434 (6×10^6 PFU in 0.5 ml of phosphate-buffered saline per 60 mm plate). It was empirically determined that the E2A mutant stocks gave the greatest number of plaques when the HeLa cell monolayers were infected with Ad5d1434 at a multiplicity of 2 PFU per cell. Under these conditions, the E2A deletion stocks formed plaques with apparent one-hit kinetics and varied in titer between 2×10^8 and 2×10^9 PFU per ml. Analysis of several of these plaques (data not shown) indicated that they contained predominately WT virus, and thus presumably arose after recombination between the two deletion mutant genomes gave rise to a WT virus. Since the helper virus assay depends on a recombination event to generate a viral plaque, it may underestimate the amount of infectious virus in the E2A mutant stocks. This does not appear to be the case, however, since a given number of E2A deletion mutant PFU contain approximately the same amount of adenovirus DNA as does the same number of WT PFU which have been

titrated in the normal plaque assay without helper virus (S. Rice, unpublished data).

Analysis of viral proteins, DNA, and RNA. The synthesis of viral proteins was assayed by labeling infected cell monolayers with 40 to 75 μ Ci of [35 S]methionine per ml and analyzing the products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (26). Immunoprecipitation of DBP and fiber protein from infected cell lysates was performed as described previously (2, 30).

Analysis of adenovirus DNA synthesis and preparation of low-molecular-weight DNA from adenovirus-infected cells have been described previously (40). To compare the numbers of adenovirus templates in infected cells, isolated nuclei remaining after cytoplasmic RNA extraction (see below) were lysed in 3.8 ml of 50 mM Tris (pH 7.4)–10 mM EDTA–100 mM NaCl–0.5% SDS, and then 0.2 ml of 10 mg of pronase per ml was added. The lysates were incubated at 37°C overnight. After two extractions with phenol-chloroform-isoamyl alcohol (24:24:1) and one extraction with chloroform-isoamyl alcohol (24:1), the nucleic acid was precipitated with 2.5 volumes of ethanol. Ten micrograms of each DNA preparation was digested with *Kpn*I, fractionated on a 1% agarose gel, and blotted to a nitrocellulose filter. The filter was hybridized with a nick-translated 32 P-labeled Ad5 DNA probe at 42°C in 50% formamide–0.4 M NaCl–0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0)–5 mM EDTA–0.2% SDS–5 \times Denhardt solution (1 \times Denhardt solution is 0.2% [wt/vol] bovine serum albumin, Ficoll [molecular weight, 400,000], and polyvinyl pyrrolidone)–100 μ g of denatured salmon sperm DNA per ml. The filters were washed twice in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, and three times in 0.1 \times SSC–0.1% SDS at 55°C.

For comparison of steady-state levels of early adenovirus mRNAs, HeLa monolayers were infected with virus at a multiplicity of 50 PFU per cell. The overlay media included 10 mM hydroxyurea (Calbiochem-Behring, La Jolla, Calif.) to block DNA replication. Cytoplasmic RNA was prepared from the cells by phenol and chloroform extraction of the 0.5% Nonidet P-40 supernatant fractions followed by ethanol precipitation (26). For Northern blot analysis, equal amounts (usually 20 μ g) of each preparation were denatured and electrophoresed on a 1.2% formaldehyde-agarose gel as described by Maniatis et al. (35). For dot blot analysis, 5 μ g of each cytoplasmic RNA preparation was denatured in formaldehyde as described above for Northern analysis, mixed with an equal volume of cold 20 \times SSC, and aspirated onto nitrocellulose through a 96-well dot blot manifold (Bethesda Research Laboratories). DNA probes specific for adenovirus early regions were labeled by nick translation with [32 P]dCTP to a specific activity of 1×10^8 to 5×10^8 cpm per μ g. Four of the probes were plasmid pBR322 derivatives containing Ad2 DNA inserts. Plasmids containing the *Ball* L (coordinates 6.0 to 7.7), *Eco*RI B (coordinates 58.5 to 70.7), and *Hind*III F (coordinates 89.5 to 97.1) fragments were probes for E1B, E2A, and E4, respectively. The plasmid pHEB4 (coordinates 0.8 to 4.5), a clone of a *Ball*-*Hpa*I double digest, was used as the probe for E1A. The purified *Eco*RI C restriction fragment from Ad5 (coordinates 75.9 to 84.0) was used as the probe for early region 3 (E3). The nitrocellulose filters were hybridized and washed as described above for the DNA blots. For quantitation of the dot blot data, individual dots were cut out and counted in 3 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.). The radioactivity bound to duplicate dots differed by

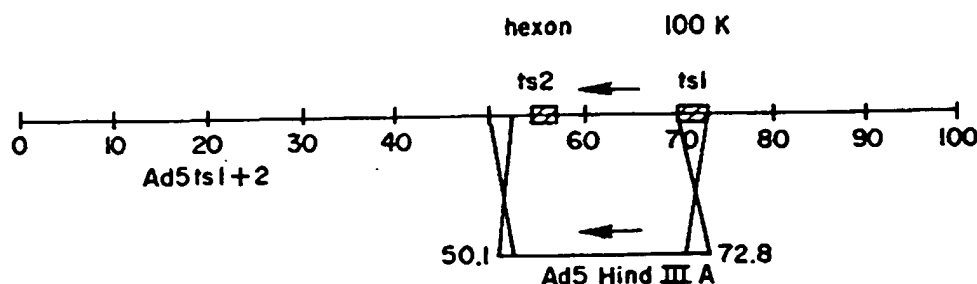


FIG. 1. Strategy for introducing a cloned E2A allele into the adenovirus genome. The upper line represents the Ad5ts1+2 genome, with the cross-hatched bars indicating the coordinates to which the ts2 hexon and ts1 100K mutations were mapped. The bottom line represents the Ad5 HindIII A restriction fragment. The arrows in the figure indicate the position and direction of transcription of the E2A gene. The two pairs of crossed lines show the recombination event which crossed out both *ts* mutations and introduced the cloned E2A allele.

less than 20%, and usually by less than 10%. The number of counts per minute binding to dots containing 5 μ g of HeLa cell RNA was considered background and was subtracted before quantitation.

RESULTS

Strategy for constructing adenovirus E2A mutants. To obtain and study DBP-defective adenovirus mutants, deletions were introduced into the E2A gene. Due to the large size of the adenovirus genome, however, it was impractical to engineer such deletions in vitro with genomic adenovirus DNA. Instead, deletions were first constructed in cloned copies of E2A, and then the altered alleles were introduced into the intact viral genomes by in vivo recombination. Our strategy for introducing a cloned E2A gene into the viral chromosome (Fig. 1) is a variation of the genetic mapping procedure called marker rescue (5, 18). In this technique a subgenomic fragment of adenovirus DNA is transfected with intact genomic DNA into permissive cells. A small but significant fraction of the progeny are recombinants which have acquired some or all of the subgenomic fragment. To select for the desired E2A recombinants, our strategy makes use of a parental virus which contains *ts* mutations in two late viral genes which flank E2A. This virus, designated Ad5ts1+2, contains both the *ts1* mutation in the 100-kDa gene (mapped between coordinates 70.0 and 71.4) and the *ts2* mutation in the hexon gene (mapped between coordinates 54.7 and 57.1) (48, 50).

The feasibility of the scheme was tested in a pilot experiment in which DNA-terminal protein complex derived from Ad5ts1+2 plus various plasmid DNAs were transfected into human 293 cells. The transfections were allowed to proceed through one infectious cycle at either the permissive or nonpermissive temperature, before a subsequent passage in HeLa cells at the nonpermissive temperature to select for non-*ts* recombinants. Only those cells which received plasmid DNA containing an adenovirus insert large enough to rescue both *ts* mutations yielded non-*ts* virus (Table 1). The rescuing plasmid, pAd5HA, contains the Ad5 HindIII A fragment (coordinates 50.1 to 72.8) cloned into the HindIII site of pBR322. This experiment also demonstrated that the state of the rescuing plasmid DNA and the conditions for selection are important. Plasmid pAd5HA DNA which had been digested with HindIII rescued much more efficiently than either undigested pAd5HA DNA or *SalI*-digested pAd5HA DNA. Since HindIII digestion releases the viral insert while *SalI* linearizes the plasmid but leaves pBR322 sequences at the ends, this result suggests that homologous ends increase the efficiency of recombination. It was also critical that the transfection be carried out at the permissive

temperature of 33°C, as no recombinants arose from transfected cells incubated at 40°C (see below).

Construction of adenovirus E2A deletion mutants. Five derivatives of the pAd5HA plasmid were constructed which contained various deletions in the DBP coding region (see above). These plasmids were designated pAd5d1801 through pAd5d1805 and are illustrated in Fig. 2. The viral inserts were released from pAd5d1801 through pAd5d1805 by HindIII digestion and then individually transfected with intact Ad5ts1+2 DNA-terminal protein complex into either 293 or dexamethasone-induced gmDBP1 cells. After one infectious cycle at 33°C, the progeny of the transfections were passaged multiple times (usually 3 days per passage) at 40°C in dexamethasone-induced gmDBP1 or gmDBP2 cells to select for non-*ts* recombinants. After three or four passages, most of the cultures which had originally been transfected with Ad5ts1+2 DNA-terminal protein complex plus the deleted E2A plasmids showed pronounced CPE, while the cultures which had originally been transfected with Ad5ts1+2 DNA-terminal protein complex alone showed no CPE. Restriction enzyme analysis of low-molecular-weight DNA isolated from these cultures indicated that the monolayers showing CPE did in fact contain adenovirus DNA, but that the E2A gene was apparently WT⁺ (i.e.,

TABLE 1. Rescue of Ad5ts1+2 by plasmid DNAs^a

Rescuing DNA ^b	Adenovirus coordinates ^c	Transfection temp (°C)	Total PFU ^d
None		33	<10
pHB1 (<i>HindIII</i> - <i>Bam</i> HI)	50.1-59.5	33	<10
pAd2HpB (<i>Bgl</i> II)	63.6-77.9	33	<10
pAd5HA (<i>Hind</i> III)	50.1-72.8	33	200,000
pAd5HA (undigested)	50.1-72.8	33	1,000
pAd5HA (<i>Sal</i> I)	50.1-72.8	33	400
pAd5HA (<i>Hind</i> III)	50.1-72.8	40	<10

^a Human 293 cells were transfected with Ad5ts1+2 DNA-terminal protein complex alone or with 10 adenovirus molar equivalents of various plasmid DNAs. The transfected cells were allowed to undergo one infectious cycle (4 days at 33°C; 3 days at 40°C). One-tenth of the progeny of the transfection was passaged in HeLa cells for 3 days at 40°C to select for non-*ts* recombinants.

^b The plasmid DNAs were first digested with the restriction enzymes indicated in parentheses. The plasmid pHB1 consists of a *HindIII*-*Bam*HI fragment of Ad2 (coordinates 50.1 to 59.5) cloned into pBR322. The plasmid pAd2HpB consists of the Ad2 *Hpa*I B fragment (coordinates 57 to 85) cloned into the *Pvu*II site of pBR322.

^c Coordinates of the potentially rescuing adenovirus fragment are indicated. For pAd2HpB, the coordinates of the internal viral fragment released by *Bgl*II digestion are indicated.

^d HeLa cell lysates were titrated on HeLa cells at 40°C to assay non-*ts* virus. The numbers shown are the total PFU in each lysate (approximately 3 \times 10⁶ cells).

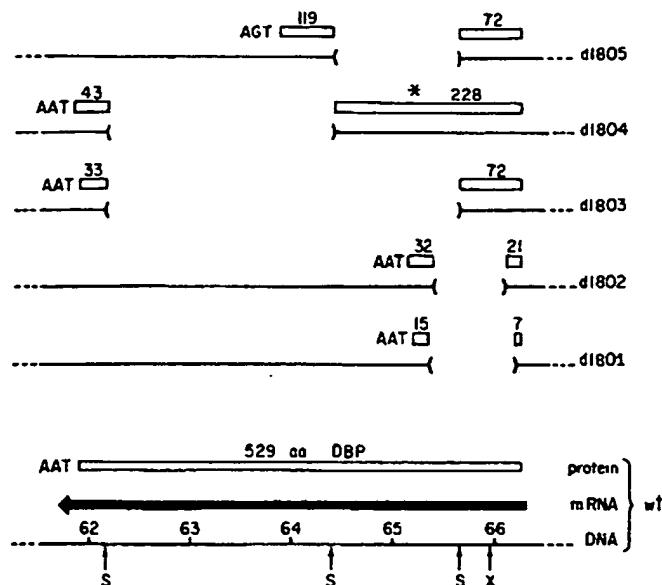


FIG. 2. Structure of the deleted E2A alleles. The lower portion of the diagram indicates the structure of the WT E2A gene, its RNA, and its protein. At the bottom, adenovirus coordinates are indicated as are the restriction sites used in the mutant construction: X denotes the Ad2 *Xho*I site at coordinate 65.9, and S denotes the Ad5 *Ssr*II sites at coordinates 62.2, 64.4, and 65.7. The large horizontal arrow indicates the coding exon of E2A mRNA, and the large open bar represents the open reading frame which encodes the 529-amino-acid (aa) DBP. The five lines above indicate the structures of the E2A genes in the deleted alleles. Parentheses denote the extent of the DNA sequences deleted, while the bars above each line represent the open reading frame predicted to be used by each mutant. Open bars correspond to translation in the normal DBP reading frame, and the stippled bars indicate that translation is in an alternate reading frame. The numbers above each bar correspond to the number of amino acids in each segment of the predicted polypeptide, while to the left of each bar is indicated the nonsense codon which should terminate translation. The asterisk above the open bar of d1804 represents the histidine to tyrosine alteration at amino acid 130 of the expected d1804 polypeptide. In the intact DBP, the alteration allows the virus to express its late genes in monkey cells.

undelated; data not shown). Southern blot analysis of the DNA preparations, however, indicated that a very small percentage of the adenovirus DNA molecules in some cultures contained the deleted E2A alleles (data not shown).

Since cells transfected with Ad5ts1+2 DNA alone never gave rise to virus after selection at 40°C, the apparent WT virus obtained after transfection with Ad5ts1+2 plus the deleted plasmid DNA must have arisen by recombination. The most probable explanation for their origin was a double recombination event or events in which plasmid DNA sequences rescued both Ad5ts1+2 *ts* mutations, but left the Ad5ts1+2 E2A gene unaltered. Although this event is probably less frequent than the desired single recombination event depicted in Fig. 1, these WT recombinants might have a significant growth advantage over recombinants containing E2A deletions, even in the induced gmDPB cell lines. Thus by the third or fourth passage the WT recombinants may be the most abundant virus in the cultures.

This hypothesis predicts that the progeny of the original transfection would include more recombinants containing the desired E2A deletion than recombinants containing the WT E2A allele. Therefore, serial fivefold dilutions of the transfection lysates were made and passaged at 40°C in the

gmDBP cell lines as described above. Most of the cultures which received the lower dilutions (5- and 25-fold) again showed pronounced CPE on the third or fourth passage and were presumed to contain WT recombinants. Cultures which received the higher dilutions (125- and 625-fold), however, appeared healthy until the fifth or sixth passage, at which time they showed definite but less pronounced CPE. Low-molecular-weight DNA was isolated from these latter cultures, digested with *Kpn*I and electrophoresed on an agarose gel. Figure 3 shows the results of this analysis for the cultures which had originally been cotransfected with pAd5d1803 through pAd5d1805. Each of these cultures appeared to contain only the desired adenovirus deletion mutant, as none of the viral DNAs contained a wild-type *Kpn*I E restriction fragment (arrow, Fig. 3). Instead, each

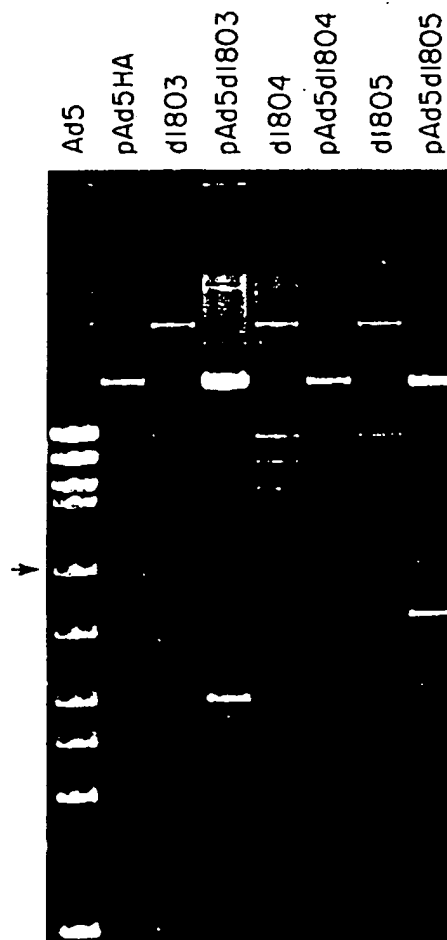


FIG. 3. *Kpn*I digestion of low-molecular-weight DNA isolated from infected gmDBP2 cells. Low-molecular-weight DNA was isolated from monolayers (60 mm) of gmDBP2 cells which were infected with WT Ad5 or with the cell lysate from fifth-passage gmDBP2 cultures originally infected with dilutions of the lysates of cells that had been cotransfected with pAd5d1803, pAd5d1804, or pAd5d1805. One-third of each DNA preparation was digested with *Kpn*I, electrophoresed on a 1% agarose gel, and stained with ethidium bromide (labeled Ad5, d1803, d1804, and d1805). To see if the viral deletions corresponded to the deletions in the parental plasmids, *Kpn*I-digested plasmid DNA (approximately 0.3 µg) from WT pAd5HA and each of the three deletion derivatives was run in the lanes immediately to the right of each corresponding viral DNA (labeled pAd5HA, pAd5d1803, pAd5d1804, and pAd5d1805). The arrow indicates the position of the wild-type *Kpn*I E restriction fragment.

restriction pattern included a smaller fragment that comigrated with the deleted *KpnI* E fragment of the parental plasmid. Similar results were obtained for the cultures which had originally been cotransfected with pAd5d1801 and pAd5d1802 (data not shown).

Large stocks of each of the five E2A deletion mutants were made in dexamethasone-induced gmDBP2 cells, starting from the dilution cultures which contained the appropriate deletion mutant DNA. The five adenovirus deletion mutants were designated Ad5d1801 through Ad5d1805. Although we did not sequence the E2A alleles of the five mutant virus, extensive restriction enzyme analysis of the viral DNAs indicated that each mutant virus had an E2A deletion consistent in structure with that of the parental plasmid (data not shown).

When the five deletion mutant stocks were titrated on HeLa cell monolayers, no viral plaques were observed. This result was consistent with the expectation that E2A defines an essential adenovirus gene. Unfortunately, no viral plaques were obtained when the deletion mutant stocks were

TABLE 2. Yields of virus in HeLa and gmDBP2a cells^a

Virus	Cell line	PFU/cell ^b
Ad5	HeLa	2.000
Ad5d1801	HeLa	0.1
Ad5	gmDBP2a	9.000
Ad5d1801	gmDBP2a	2
Ad5d1801	gmDBP2a (+ dex) ^c	90

^a Confluent monolayers were infected at a multiplicity of 20 PFU per cell and incubated for 68 h at 37°C.

^b Progeny virus were harvested by freeze-thawing and sonication. The yields of Ad5 were determined by titration on HeLa cells. The yields of Ad5d1801 were determined by titration on HeLa cells using Ad5d1434 as a helper virus.

^c The overlay media included 0.6 μ M dexamethasone (dex).

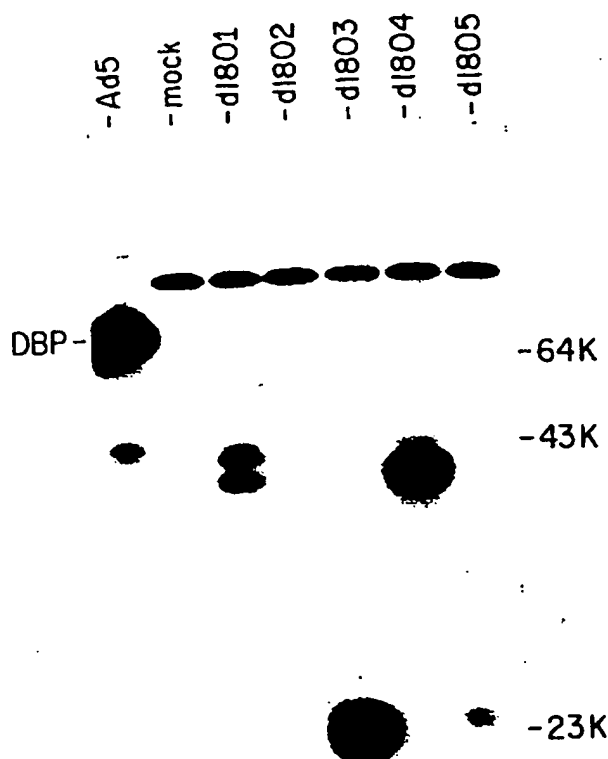


FIG. 4. DBP-related polypeptides synthesized in E2A deletion mutant-infected HeLa cells. Confluent monolayers of HeLa cells (60 mm) were infected at a multiplicity of 100 PFU per cell with WT Ad5 or with Ad5d1801 through Ad5d1805. At 20 h p.i. the monolayers were labeled for 1 h with 75 μ Ci of [³⁵S]methionine per ml. One-fourth of each labeled protein extract was immunoprecipitated with polyclonal rabbit antisera raised against purified DBP, and the immunoprecipitates were electrophoresed on a 15% SDS-polyacrylamide gel. An autoradiogram of the gel is shown. To the right of the panel, the position at which molecular weight marker proteins migrated is indicated (in thousands [K]). The position of the 72-kDa DBP is indicated to the left of the panel. The approximately 100-kDa protein which is seen in most lanes is a cellular protein which is sometimes immunoprecipitated by the anti-DBP serum.

titrated on gmDBP1 or gmDBP2 cells in the presence of dexamethasone, even though these monolayers efficiently supported plaque formation by WT virus (see below). In an attempt to assay the infectivity of the deletion mutants, we titrated the mutant stocks on HeLa cells in the presence of a helper virus (see above). The helper virus used was Ad5d1434, an E1A-E1B deletion mutant (lacking sequences between viral coordinates 2.6 and 8.7; D. Solnick, unpublished data) which is itself defective for plaque formation and growth in HeLa cells. When HeLa cell monolayers were uniformly infected with Ad5d1434 at a multiplicity of 2 PFU per cell, the E2A deletion mutant stocks formed plaques with apparent one-hit kinetics and varied in titer between 2×10^8 and 2×10^9 PFU per ml.

DBP-related polypeptides encoded by the E2A deletion mutants. Since the DNA sequence of both the Ad2 and Ad5 E2A genes is known (32, 33), it is possible to predict the effects of the deletion mutations on E2A coding potential (Fig. 2). Four of the five deletions cause frameshift mutations in the DBP-coding region. The exception is the Ad5d1803 deletion which fuses in frame amino-terminal and carboxyl-terminal coding regions. To test these predictions, HeLa cell monolayers were infected with WT or mutant virus, and at 24 h p.i. they were pulse-labeled with [³⁵S]methionine. The labeled protein extracts were immunoprecipitated with a polyclonal antiserum raised against purified DBP (Fig. 4). As expected, none of the mutant-infected HeLa cells synthesized an intact 72-kDa DBP. With one exception, the mutants produced DBP-related proteins that were consistent in size with the predictions shown in Fig. 2. The exception was Ad5d1801, which was predicted to encode a 22-amino-acid polypeptide. Unexpectedly, Ad5d1801-infected cells synthesized two DBP-related polypeptides of 40 and 37 kDa. The origin of these Ad5d1801 proteins is not understood at this time (see below). Ad5d1802 was predicted to encode a small (47 amino acid) and probably undetectable DBP-related protein. Consistent with this, no detectable protein was immunoprecipitated from the Ad5d1802 infections. HeLa cells infected with Ad5d1803 through Ad5d1805 synthesized DBP-related proteins of approximately 17, 38, and 18 kDa, respectively, which are in reasonable agreement with the predicted 12-, 30- and 21-kDa polypeptides. These sizes are not inconsistent with expectation, since the WT DBP has a molecular weight of 59 kDa, as deduced by its amino acid sequence, but migrates on SDS-polyacrylamide gels as a 72-kDa protein, possibly as a result of the high proline content near the amino terminus of DBP (32, 33). In some additional experiments, the DBP-related proteins encoded by Ad5d1803 and Ad5d1804 were resolved into triplet and doublet species, respectively (data not

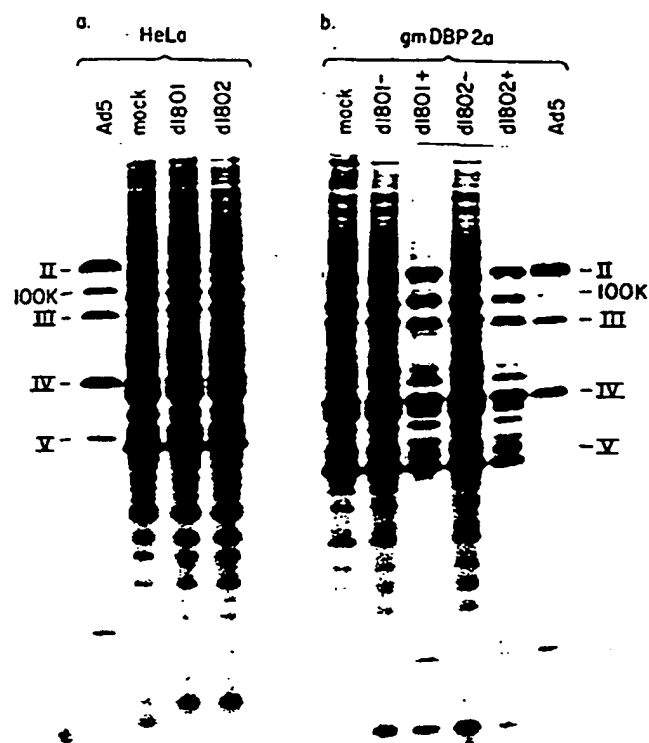


FIG. 5. Viral protein synthesis in E2A deletion mutant-infected cells. Confluent monolayers of HeLa cells (a) or gmDBP2a cells (b) were infected at a multiplicity of 20 PFU per cell with WT Ad5, Ad5d1801, or Ad5d1802. Mutant-infected gmDBP2a cells were incubated either in the absence (-) or presence (+) of 0.6 μ M dexamethasone. At 40 h p.i., the monolayers were labeled for 1 h with 40 μ Ci of [35 S]methionine per ml. Equal fractions of each cell lysate were electrophoresed on a 15% SDS-polyacrylamide gel, and the dried gel was subjected to autoradiography. The positions of several late adenovirus proteins are indicated.

shown), but the origin of this protein heterogeneity has not been investigated.

E2A deletion mutants are defective for viral growth and DNA replication. As mentioned above, none of the five E2A deletion mutant stocks formed plaques on HeLa cells, suggesting that these mutants were defective for viral growth. To test this directly, monolayers of HeLa or gmDBP2a cells (a subclone of the gmDPB2 cell line) were infected with WT Ad5 or Ad5d1801, and the infections were allowed to proceed through an infectious cycle at 37°C. Virus yields from these infections are shown in Table 2. As expected, Ad5d1801 is defective for growth in HeLa cells, producing less than 1 PFU per infected cell. Although a small amount of progeny Ad5d1801 virus were produced in gmDBP2a cells in the absence of dexamethasone (2 PFU per cell), hormone stimulation was essential for efficient propagation of this mutant (90 PFU per cell). Note that even with dexamethasone stimulation, Ad5d1801 grew approximately 100-fold less efficiently in gmDBP2a cells than did WT Ad5.

Growth of adenovirus can also be evaluated by monitoring late viral protein synthesis. Therefore, monolayers of HeLa or gmDBP2a cells were infected with WT Ad5, Ad5d1801, or Ad5d1802 and pulse-labeled for 1 h with [35 S]methionine at 40 h p.i. The labeled proteins were electrophoresed on an SDS-polyacrylamide gel to assay for the synthesis of late viral proteins (Fig. 5). No late viral proteins could be detected in either mutant-infected HeLa cells or mutant-

infected gmDBP2a cells in the absence of dexamethasone. When gmDBP2a cells were infected with Ad5d1801 or Ad5d1802 in the presence of dexamethasone, however, synthesis of late viral proteins was easily detected, although shut-off of host protein synthesis did not occur as efficiently as in the corresponding WT infections. Similar results were obtained for mutants Ad5d1803 through Ad5d1805 (data not shown).

Since DBP is known to be required for adenovirus DNA synthesis (16, 47), it was strongly suspected that the E2A deletion mutants were defective for viral DNA replication. To test this, HeLa or dexamethasone-induced gmDBP2a monolayers were infected with WT Ad5, Ad5d1801, or Ad5d1802, and labeled from 28 to 34 h p.i. with [3 H]thymidine. Low-molecular-weight DNA was extracted from the cells, digested with *Kpn*I, and electrophoresed on an agarose gel. No viral DNA synthesis could be detected in mutant-infected HeLa cells (Fig. 6), even after very long exposures of the fluorogram. The two deletion mutants did replicate their DNA in gmDBP2a cells in the presence of dexamethasone, but at a reduced rate (approximately 30- to 50-fold) compared with that of WT. Although the mutants Ad5d1803 through Ad5d1805 were not directly tested for viral DNA replication, they were probably also defective since they failed to produce the intact carboxy-terminal segment of the DBP which contains in vitro DNA replication activity (4).

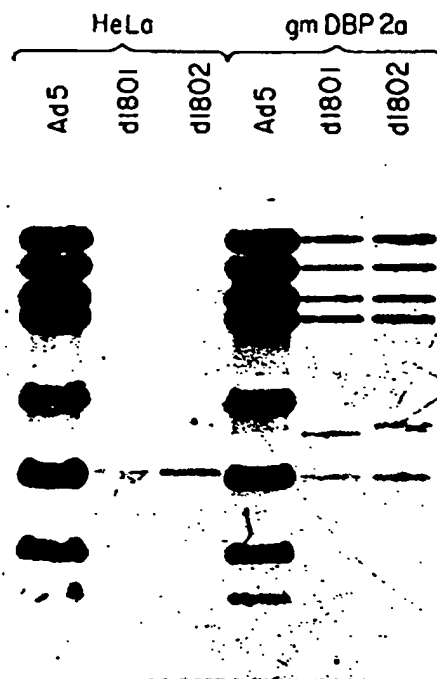


FIG. 6. Viral DNA synthesis in E2A deletion mutant-infected cells. Confluent monolayers (60 mm) of HeLa or gmDBP2a cells were infected at a multiplicity of 20 PFU per cell with WT Ad5, Ad5d1801 or Ad5d1802. The gmDBP2a infections were incubated in media containing 0.6 μ M dexamethasone. The monolayers were labeled from 28 to 34 h p.i. with [3 H]thymidine, and low-molecular-weight DNA was isolated. One-fourth of each DNA sample was digested with *Kpn*I and electrophoresed on a 1% agarose gel. Shown is an autoradiogram of the dried gel after treatment for fluorography. The labeled band comigrating with the Ad5 *Kpn*I F fragment was seen in mock-infected cells and probably represents synthesis of mitochondrial DNA.

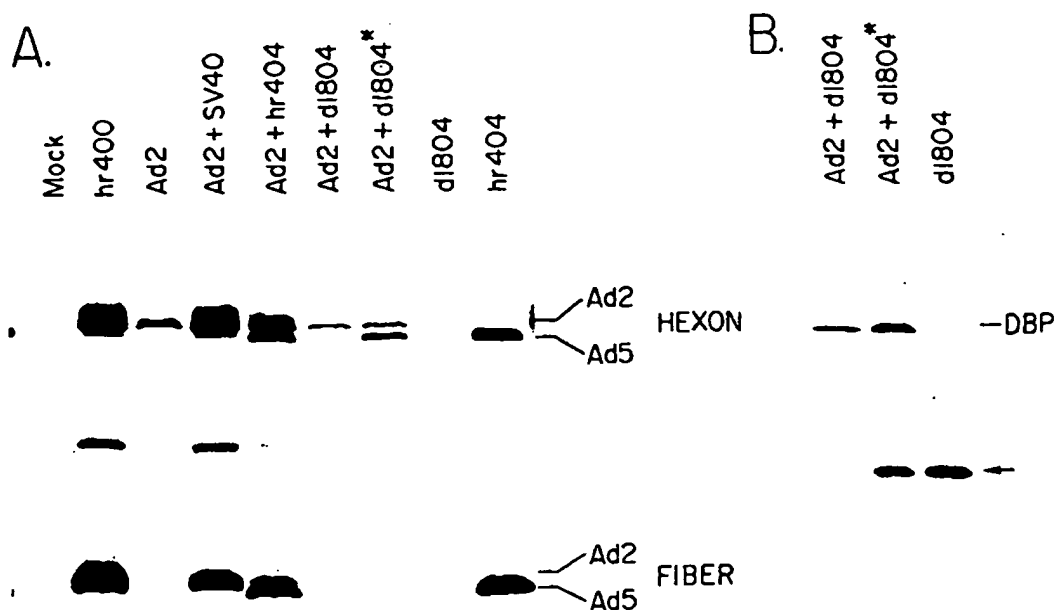


FIG. 7. Failure of the Ad5d1804 DBP-related polypeptide to enhance late viral gene expression in monkey cells. Monolayers of CV₁ cells were either singly infected (40 PFU per cell) or doubly infected (20 PFU per cell of each virus except for one infection [indicated by an asterisk] in which 20 PFU of Ad2 per cell and 100 PFU of Ad5d1804 per cell were used) with adenovirus mutants. At 36 h p.i. the monolayers were labeled for 2 h with 40 μ Ci of [³⁵S]methionine per ml. Equal fractions of each labeled protein extract were immunoprecipitated with polyclonal rabbit antisera raised against purified native fiber protein (A) or purified native DBP (B). In panel A, note that the Ad2 and Ad5 fiber polypeptides can be distinguished, as can Ad2 and Ad5 hexon polypeptides which are also partially immunoprecipitated by the fiber antiserum. In panel B, the positions of the 72-kDa DBP and the Ad5d1804-specific, DBP-related polypeptide are indicated (arrow). SV40 coinfection also allows WT adenovirus to grow productively in monkey cells (8).

Expression of the truncated DBP-related polypeptide of Ad5d1804 does not enhance late viral gene expression in monkey cells. We have previously presented data which suggests that the amino-terminal domain of DBP may act as an independent functional domain which can enhance late viral gene expression and viral growth in monkey cells (40). If so, then the DBP-related polypeptide encoded by Ad5d1804 (Fig. 2) may retain this function since this truncated polypeptide should contain an amino-terminal domain derived from Ad5hr404, an E2A mutant able to express its late genes in monkey cells (29, 32). If the Ad5d1804 DBP-related polypeptide is functional, then Ad5d1804 should enhance WT Ad2 late gene expression when both viruses are coinfecting into monkey cells. Since Ad2 is not defective for DNA replication in monkey cells, the coinfecting cells should progress into the late phase of infection in which the DBP fragment containing the *hr* mutation might help overcome the block to late gene expression. Therefore, confluent monolayers of CV₁ cells, a line of African green monkey kidney cells, were infected with various virus inocula, incubated until well into the late phase (36 h p.i.), and then labeled for 2 h with [³⁵S]methionine. Although synthesis of most late viral proteins is depressed in abortively infected monkey cells, the fiber polypeptide is the most dramatically affected, being reduced some 100- to 200-fold (2, 26). The labeled protein extracts therefore were immunoprecipitated with a polyclonal antibody prepared against purified fiber protein, and the immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel (Fig. 7A). Note that Ad2 and Ad5 fiber polypeptides can be distinguished in this gel system, as can Ad2 and Ad5 hexon polypeptides which are also partially precipitated with the fiber antiserum. As expected, Ad2-infected CV₁ cells produced very little fiber

compared with that of productive CV₁ cell infections (Ad2-SV40, Ad2hr400, and Ad5hr404). As a positive control, CV₁ cells were coinfecting with Ad5hr404 and Ad2, and as expected the hr404 DBP functioned *in trans* to enhance fiber synthesis. Ad5d1804, however, could not enhance either fiber or hexon synthesis, even when very high Ad5d1804 multiplicities of infection (100 PFU per cell) were used (asterisk, Fig. 7). Lack of enhancement was not due to the failure of the coinfecting cells to express the Ad5d1804 DBP-related protein, as it was easily detected when the labeled extracts were immunoprecipitated with anti-DBP serum (Fig. 7B). However, immunoblot analysis of the protein extracts demonstrated that the steady-state levels of the Ad5d1804 polypeptide were reduced between 10- and 40-fold in the coinfections compared with the level of DBP in a WT infection (data not shown). Determination of virus yields of parallel infections showed that Ad5d1804 could not enhance Ad2 growth in CV₁ cells (data not shown), which is consistent with its inability to enhance fiber synthesis. Very similar results were obtained when these experiments were repeated with CV₁ cells (data not shown), a monkey cell line in which the block to Ad2 growth is less stringent than in CV₁ cells (40).

The role of DBP in adenovirus early gene regulation. To study the role of DBP in viral early gene regulation, steady-state levels of early mRNAs were monitored in WT Ad5-, Ad5ts125-, and Ad5d1802-infected HeLa cells. Since Ad5d1802 produces no DBP or detectable DBP fragment, this experiment allows one to observe adenovirus early gene expression in the complete absence of any DBP-mediated regulation. HeLa monolayers were infected at a multiplicity of infection of 50 PFU per cell with each virus and incubated at 40°C. To validate comparisons between WT Ad5 and the

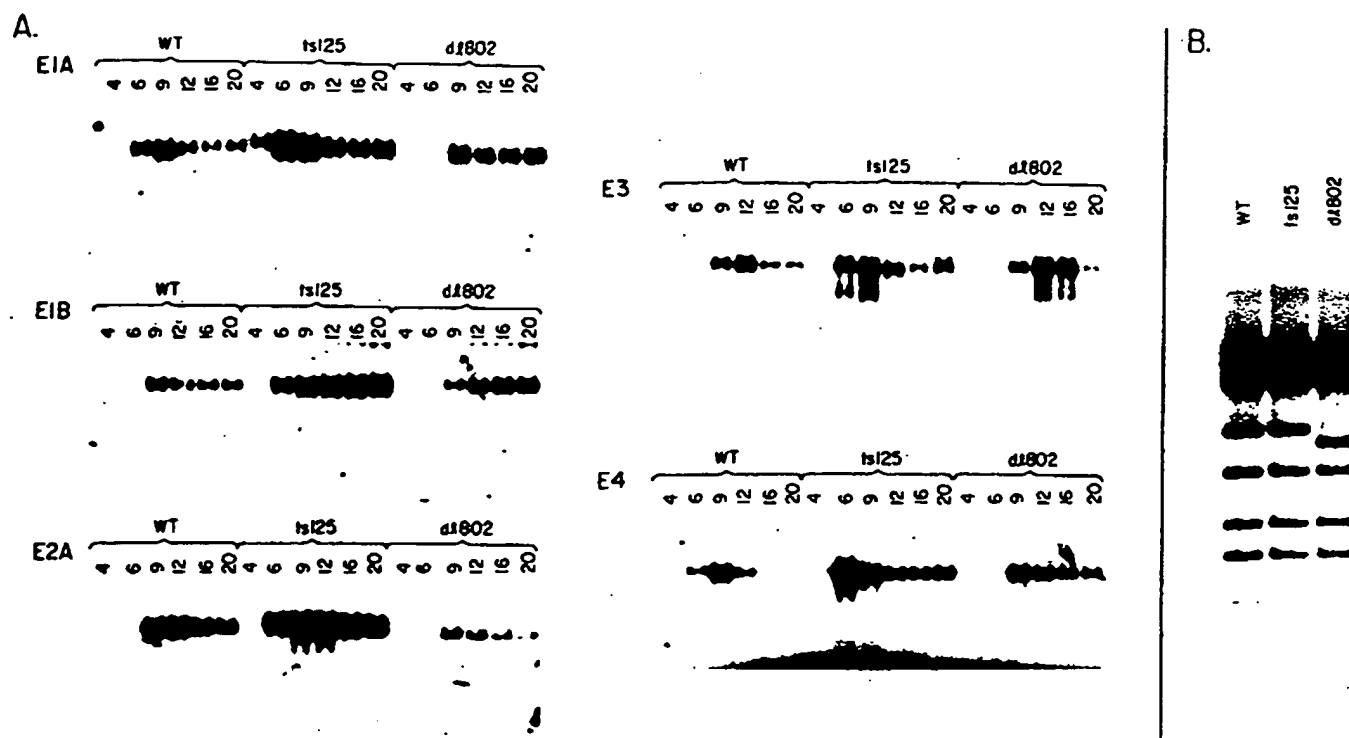


FIG. 8. Analysis of early adenovirus gene expression in DBP mutant-infected cells. Confluent monolayers of HeLa cells were infected at a multiplicity of 50 PFU per cell with WT Ad5, Ad5d1802, or Ad5ts125. The infections were incubated at 40°C in media containing 10 mM hydroxyurea. (A) Northern blot analysis of early viral RNA expression in mutant-infected cells. At various times after infection (indicated in hours by the number immediately above each lane) cytoplasmic RNA was extracted. Equal amounts of each RNA preparation were denatured, electrophoresed on a 1.2% formaldehyde-agarose gel, and subjected to Northern blot analysis with 32 P-labeled DNAs specific for various early regions (indicated next to each panel) as probes. (B) Comparison of the number of viral templates in infected cell nuclei. Total nuclear DNA was prepared from the cells used for the cytoplasmic RNA extractions at 20 h p.i. Ten micrograms of each DNA preparation was digested with *Kpn*I and subjected to Southern blot analysis with 32 P-labeled Ad5 DNA used as a probe.

two DNA replication-defective mutants, the potent DNA replication inhibitor hydroxyurea was added to all the infections. At various times after infection, cytoplasmic RNA was prepared from the infected cells. As a control, to make sure that there were approximately equal numbers of viral templates in each infection, nuclear DNA was prepared from the infected cells at the last time point (20 h p.i.). Equal amounts of the DNA preparations were digested with *Kpn*I and subjected to Southern blot analysis with 32 P-labeled Ad5 DNA as a probe (Fig. 8B). This analysis confirmed that there were approximately equal numbers of viral DNA molecules in each infected cell nucleus. To compare the steady-state levels of messages from various early regions, equal amounts of the cytoplasmic RNA preparations were subjected to Northern blot analysis with 32 P-labeled DNAs specific for E1A, E1B, E2A, E3, and E4 as the hybridization probes (Fig. 8A).

Three general conclusions can be drawn from the results of this experiment and two repeat experiments of the same design (data not shown). First, DBP does not appear to be a significant positive regulator of either E1B or E4 mRNA expression in infected HeLa cells, since WT and Ad5d1802 show similar patterns for both the induction and continued expression of these two early genes. Second, we observed that E2A mRNA levels were reduced significantly in Ad5d1802 infections (Fig. 8A). Also note that E2A mRNA from Ad5d1802 infections migrates slightly faster than does E2A mRNA from WT infections, consistent with the expectation that it contains a 242-nucleotide internal deletion. Quantitation of mRNA levels by dot blot hybridization

analysis (data not shown) indicated that E2A mRNAs were reproducibly reduced about fivefold in Ad5d1802 infections as compared with WT Ad5 infections. These results are consistent either with a positive role for DBP in the *trans*-acting regulation of its own gene or with a *cis*-acting defect caused by the Ad5d1802 deletion. Third, DBP does not appear to negatively regulate expression of E1A, E1B, E3, or E4 mRNAs when DNA replication is tightly blocked since Ad5d1802 shows little if any overexpression of these regions when compared with that of WT Ad5. In some experiments (e.g., E4 in Fig. 8A), slight overexpression (approximately twofold) of early mRNAs in Ad5d1802-infected cells was noted, but these results were not reproducible in other experiments. Ad5ts125-infected cells, however, did show a reproducible tendency to overexpress early mRNAs at late times (12 to 20 h p.i.) when compared with that of WT Ad5 (e.g., E1B in Fig. 8A). The observed effect, however, was quite variable and never dramatic (maximally about fourfold). Much more reproducible was the tendency for Ad5ts125-infected cells to express early viral genes sooner than WT Ad5 or Ad5d1802-infected cells and at higher levels at the earlier times (4 to 9 h p.i.; this can be observed for all early regions in Fig. 8A). Since the DBP-negative mutant Ad5d1802 behaves much more similarly to WT Ad5 than to Ad5ts125, we conclude that DBP does not have a significant negative effect on the accumulation of E1A, E1B, E3, or E4 mRNAs in infected HeLa cells monolayers when DNA replication is tightly blocked. No conclusions can be drawn concerning the negative regulation of E2A by DBP, since Ad5d1802 fails to properly initiate E2A expression.

DISCUSSION

In this report a genetic system is described which can be used to construct Ad5 mutants of any desired E2A genotype. The E2A mutations were first engineered into a cloned E2A gene by techniques of *in vitro*, site-directed mutagenesis. Next the cloned, mutated alleles were transfected into human cells with genomic DNA-terminal protein complex derived from an adenovirus mutant, Ad5ts1+2, which contains *ts* mutations in two genes flanking E2A. Homologous recombination occurs in the transfected cells, creating viral progeny which crossed out both *ts* mutations and crossed in the mutant E2A allele. These recombinants can be genetically selected and propagated by growth at the nonpermissive temperature in DBP-producing cell lines (27). This protocol was used to isolate and propagate five Ad5 E2A deletion mutants, designated Ad5d1801 through Ad5d1805.

In the course of these experiments, it was discovered that the transfections need not be carried out in the DBP-producing cell lines. Four of the five deletion mutants described here were isolated after transfection of the noncomplementing but highly transfectable 293 cell line. Our results are most easily explained by assuming that recombinants constitute only a minor fraction of the viral templates in any given transfected cell. Thus the E2A mutants need not be complemented by the cell line since they are efficiently complemented by the DBP produced by the many Ad5ts1+2 templates. Consistent with this model is the observation that no recombinants were recovered when the transfection protocol was carried out at the nonpermissive temperature (Table 1). Recombinant genomes probably are formed at the nonpermissive temperature, but are not packaged due to the large amount of defective 100K and hexon polypeptides produced by the numerous Ad5ts1+2 templates.

Although the protocol described allowed the construction of several E2A mutants, two factors limit its general efficiency as a means of isolating large numbers of DBP mutants. First, the system requires the E2A mutations be introduced into the pAd5HA plasmid, since it is the smallest E2A-containing plasmid which can also rescue both mutations of Ad5ts1+2. The pAd5HA plasmid, however, is relatively large and therefore is not easily manipulated *in vitro*. We currently are testing other strategies for introducing a cloned E2A allele into the adenovirus genome which may allow us to use smaller E2A-containing plasmids. The second factor which has limited the utility of this system is the fact that E2A-defective mutants fail to form viral plaques on the complementing gmDBP cell lines. This failure is probably due to the relatively inefficient growth of the E2A mutants in the gmDBP cell lines (see Table 2). Since several rounds of viral replication normally are required to create a visible adenovirus plaque, the reduced yield of the mutants combined with the limited viability of the gmDBP monolayers during a plaque assay (about 9 days at 37°C) is probably responsible for the inability to detect plaques.

The failure of the DBP-defective mutants to form plaques results in two important problems with respect to the study of these mutants. First, the number of infectious virus in a mutant stock cannot be directly titrated in a plaque assay. Fortunately, we obviated this problem by titrating the mutant stocks on HeLa cells using a helper virus. The second and more serious problem is that, without plaque formation, E2A mutants cannot be directly isolated from a virus population. This is particularly serious since rescue of Ad5ts1+2 with E2A deletion plasmids often creates a small class of double recombinants which are WT for E2A and can rapidly

outgrow the desired E2A mutants. Plaque formation would also allow the transfection protocol to be carried out with a collection of mutagenized plasmids, rather than a single mutant plasmid. Large numbers of individual viral mutants could be isolated from a single mutagenesis and transfection experiment and screened for desired phenotypes.

The E2A deletions in Ad5d1801 and Ad5d1802 extended very near to the amino terminus of DBP-coding sequences. Since these deletions also caused frameshift mutations, both mutants were predicted to encode small proteins with very limited identity to DBP. This expectation was upheld for the mutant Ad5d1802, which made no detectable DBP-related polypeptide and thus represents the first DBP-negative adenovirus mutant. Ad5d1801, however, unexpectedly produced two DBP-related proteins of approximately 40 and 37 kDa. Although we have not yet investigated the origin of these proteins, it is interesting that Asselbergs et al. (6) have identified rare E2A mRNAs which can be translated *in vitro* into DBP-related proteins of similar size.

All five of the E2A deletion mutants appear to be absolutely defective for growth in HeLa cells, thus verifying the expectation that DBP is an essential adenovirus protein. It was also demonstrated that Ad5d1801 and Ad5d1802, and probably Ad5d1803 through Ad5d1805, are completely defective for viral DNA replication. Since most previously isolated adenovirus mutants which are DNA replication defective are to some extent leaky, it seems likely that the E2A deletion mutants described here are the tightest DNA replication-negative mutants in existence. As such, they may be useful for the study of the regulation of those adenovirus functions, such as late gene expression (44), which are dependent on viral DNA replication.

The *hr* mutant DBP carries out an activity in monkey cells which enhances the expression of late viral genes (29). The large T antigen of SV40 is functionally similar to DBP in that it can also enhance late adenovirus gene expression in monkey cells (8). In the case of T antigen, the enhancement activity is carried out by an independent functional domain at the carboxyl terminus of the protein, since this domain can function even when attached to other polypeptides (15, 45). We have suggested previously that the amino-terminal portion of DBP defines an analogous, and perhaps independent, functional domain (40). Evidence for this came from the study of the E2A double mutant Ad2ts400 which contains both an amino-terminal *hr* mutation and a carboxy-terminal *ts* mutation. Ad2ts400 was able to enhance late viral gene expression in monkey cells at the nonpermissive temperature, even when the Ad2ts400 DBP was nonfunctional for viral DNA replication. The mutant Ad5d1804 was constructed to test directly whether an amino-terminal portion of DBP containing the *hr* mutation could function as an independent domain. This mutant encodes a protein in which the amino-terminal 228 amino acids of the Ad5hr404 DBP are fused to 43 amino acids translated from an alternate reading frame. When Ad5d1804 was coinfecting with Ad2 into monkey cells, however, enhancement of late viral gene expression did not occur. Several interpretations of this result are possible. First, the amino-terminal portion of DBP may not define a functionally independent domain, requiring some or all of the carboxy-terminal portion for activity. Alternately, the amino-terminal portion may define an independent functional domain, but the particular conformation of the Ad5d1804 polypeptide, perhaps because of its abnormal carboxy terminus, renders this particular protein nonfunctional. Another possibility which cannot be ruled out is that the Ad5d1804 protein failed to enhance late gene ex-

pression because it was not produced in sufficient quantities. Indeed, immunoblot analysis indicated that the steady-state level of the Ad5d1804 protein in the coinfecting cells was reduced approximately 10- to 40-fold compared with the level of DBP in a WT infection. Additional DBP mutants, which efficiently express only the N-terminal domain, will be required to distinguish among these possibilities.

Isolation of the DBP-negative mutant Ad5d1802 allowed us to study adenovirus early gene expression in the absence of any DBP-mediated regulation. Results of previous studies which involved the microinjection of purified adenovirus genes or mRNAs into mammalian cells suggest that DBP may be required for or have a stimulatory effect on the expression of E1B (42) and E4 (41). Results of our experiments, however, indicate that DBP does not positively regulate the mRNAs of these genes in infected HeLa cells. It is important to note that since both microinjection studies assayed gene expression at the protein level, it remains possible that DBP stimulates translation of E1B and E4 mRNAs in HeLa cells.

These experiments unexpectedly show that Ad5d1802 fails to normally express E2A, as steady-state levels of Ad5d1802 E2A mRNAs were consistently reduced approximately fivefold compared with that of WT infections. Two hypotheses can be proposed to explain these data. First, DBP may be a positive *trans*-acting regulator of its own gene. Second, and perhaps more likely, is the possibility that the E2A deletion in Ad5d1802 causes a *cis*-acting defect which prevents efficient E2A expression. The sequences removed by the deletion may be required for efficient E2A transcription or RNA processing, or perhaps the altered Ad5d1802 E2A mRNA has a shorter half-life. Preliminary analysis has indicated that Ad5d1801-infected HeLa cells exhibit a similar deficiency in the expression of E2A mRNA.

Results of studies with *ts* E2A mutants such as Ad5ts125 have suggested that DBP negatively regulates expression from several early regions, since Ad5ts125-infected cells accumulate abnormally high levels of early mRNAs at intermediate and late times after infection (7, 10, 11, 39). In our experiments overaccumulation of E1A, E1B, E2A, E3, and E4 mRNAs was sometimes observed in Ad5ts125-infected cells at later times (12 to 20 h p.i.), but the effect was variable and never dramatic. A more reproducible effect of the Ad5ts125 mutation was the tendency of Ad5ts125-infected cells to express early mRNAs sooner than WT Ad5 and in higher amounts at these early times (4 to 9 h p.i.). Although we do not understand the discrepancy between these and previous results concerning Ad5ts125, perhaps variables such as the cell lines used are important. These studies used monolayer cultures of HeLa cells while previous studies which reported larger differences used HeLa cell suspension (7, 40) or KB suspension (10, 11) cultures. It may also be important that we used hydroxyurea to inhibit viral DNA replication, since cytosine arabinoside, the drug used in previous studies, in our hands often was not completely effective at inhibiting DNA synthesis. Repression of early gene expression by DBP may depend on a low level of DNA replication or certain physiological conditions which may occur in the presence of cytosine arabinoside but not in the presence of hydroxyurea.

If DBP were an important negative regulator of early mRNA levels, one would expect that the DBP-negative mutant Ad5d1802 would have a more dramatic phenotype, i.e., different from that of WT Ad5, than the *ts* DBP mutant Ad5ts125. This was not the case. Although Ad5d1802-infected HeLa cells sometimes showed slightly enhanced

levels of early mRNAs compared with WT-infected cells, most often little (<2-fold) or no difference was observed. In no experiment did the DBP-negative mutant accumulate higher amounts of early mRNAs than did Ad5ts125. While it is impossible to exclude subtle effects of DBP, we conclude that DBP does not play a significant role in the negative regulation of E1A, E1B, E3, or E4 mRNA levels in infected HeLa cell monolayers when DNA replication is tightly blocked.

The E2A mutants Ad5ts125 and Ad5d1802 thus appear to differ somewhat in phenotype with respect to early gene expression. However, since the overaccumulation of early RNAs observed in Ad5ts125-infected cells was not dramatic and quite variable, we do not wish to overemphasize this difference between the two mutants. Ad5ts125 and Ad5d1802 can be easily distinguished with respect to another phenotype, however. At 38.5°C Ad5ts125 transforms rat embryo cells at a three- to eightfold enhanced rate compared with that of WT virus (19, 49), but Ad5d1802 as well as the other E2A deletion mutants transform at the same rate as WT (S. Rice, D. Klessig, and J. Williams, unpublished data). While it is not immediately apparent why an E2A *ts* mutant should have a phenotype not possessed by an E2A deletion mutant, it may reflect the multifunctional nature of DBP. Perhaps the temperature-sensitive DBP acquires altered and even pathological activities at the nonpermissive temperature because of the differential inactivation of functional domains.

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CLONING AND EXPRESSION OF GLYCOPROTEIN GENES IN HUMAN ADENOVIRUS VECTORS¹

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ABSTRACT Adenovirus vectors have proven useful for achieving high level expression of a variety of foreign genes and have many features which make them attractive for use as recombinant viral vaccines. The human adenovirus genome contains at least two regions into which DNA can be inserted to generate helper independent vectors: early region 3 (E3) which is nonessential for replication of virus in cultured cells, and E1, which is nonessential for replication in 293 cells which provide E1 functions in trans. We have developed novel approaches for rescue of insertions into both E1 and E3 and have used these methods as well as conventional techniques to construct vectors expressing dihydrofolate reductase, Herpes Simplex Virus thymidine kinase (HSV TK), Vesicular Stomatitis Virus glycoprotein G (VSV G), and HSV glycoprotein B (HSV gB) and HSV gC, as well as the glycoprotein encoded by rabies virus. Both the VSVG and HSVgB recombinants produced high levels of the corresponding glycoprotein in infected human cells and cells of other species. The VSV vector elicits neutralizing antibody in a variety of different animals including pigs, dogs, cows and mice. The HSV gB expression vector has been shown not only to induce production of circulating antibody in infected mice, but to be able to protect mice against a subsequent challenge with HSV.

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INTRODUCTION

An exciting prospect arising from the application of recombinant DNA technology to the diagnosis and prevention of disease is the development of mammalian virus vectors for delivery and expression of antigens in suitable host cells or for use as live virus vaccines. An ideal vector is a non-pathogenic virus whose genome allows insertion of foreign genes under the control of appropriate promoters and allows subsequent delivery and expression of the foreign genes in the desired host. For example, the development and characterization of vaccinia virus vectors is well advanced and their efficacy in animal immunization is well documented (1,2). Although the problem of vaccinia virus toxicity still mitigates against their quick acceptance as human and animal vaccines (3), great strides have been made in modifying the virus to reduce toxicity and the vaccinia system has amply demonstrated the potential of virus vector vaccines. It is highly unlikely that a single vector system will suffice as a vaccine in all situations and for all pathogens. As work with recombinant viruses progresses, numerous factors including the stability of the vector, ease in administration, site of replication, as well as the replicative biology of the vector, will all play a part in determining the utility of this approach. Human adenoviruses have a number of advantages as potential virus vaccine vectors and as expression vectors for the study of proteins of other organisms. Their genome is reasonably large, 30-40kb, so that genes encoding an average sized protein can be inserted into nondefective vectors. Adenoviruses are able to express large amounts of virus coded proteins in infected cells, offering the potential for expression of correspondingly large amounts of foreign gene products, and human adenoviruses have been extensively characterized both genetically and biochemically (4). From the point of view of vaccine development it is noteworthy that a live vaccine consisting of human adenoviruses types 4 and 7 has been used extensively by the US military since the early 1970's (5).

Two regions of the Ad5 genome are available as insertion sites for foreign DNA sequences. One of these is E3 which extends from 76.6mu to 86.0mu and is nonessential for viral replication in cultured cells, and the other is E1 (1.3-11.2mu) which is nonessential for viral replication in 293 cells (6). E3 contains two convenient XbaI sites at 78.5mu and 84.7mu which can be used to delete 1880bp and to provide a restriction site for insertion of foreign DNA sequences. The

resulting vectors, though lacking E3 functions, are capable of replicating in cultured cells as well as in vivo (7). An advantage of insertions in region E1 is that these render the virus defective (limited to replication in 293 cells) which provides good biocontainment for work with vectors expressing potentially pathogenic antigens. Deletion of E1 sequences and substitution of foreign DNA results in a highly attenuated expression vector capable of only very limited replication in normal human cells, a property which may have advantages for certain applications.

RESULTS

The methods used for insertion of foreign DNA into E1 or E3 are basically similar. Briefly, the gene to be inserted is trimmed so that there are no initiation codons upstream of the genuine site of initiation and the coding sequences are inserted into an expression cassette which for many of our constructs consisted of the SV40 early promoter, a polycloning site, and the transcription termination and polyadenylation signals from the SV40 large T Ag gene. This combination of control elements was chosen on the assumption that the SV40 early promoter would drive expression of genes inserted into either E1 or E3. Subsequent analyses, as discussed below, suggested that at least for some inserts in E3 the SV40 sequences did not function as a strong promoter but may serve as a splice acceptor for transcripts originating in upstream adenovirus sequences. For some expression vectors we utilized controlling sequences other than those of SV40. The cassette is flanked on either side by XbaI sites which are used to transfer the cassette into XbaI cloning sites in either E1 or E3 of appropriate Ad5 vectors. Actual rescue of the insert into the genome of infectious virus can be achieved by a variety of methods. These can be broadly grouped into two classes: conventional techniques utilizing DNA extracted from purified virions (cf Fig. 1), or novel techniques we have developed utilizing bacterial plasmids containing the entire Ad5 genome in infectious form (cf Figs. 2-3).

Vector Construction Using Infectious Adenovirus Plasmids

We have shown that adenovirus DNA circularizes in infected cells (8) and have used this phenomenon to isolate bacterial plasmids which contain the entire Ad5 genome with ends covalently joined, and which are able to regenerate infectious virus following transfection of human cells (9).

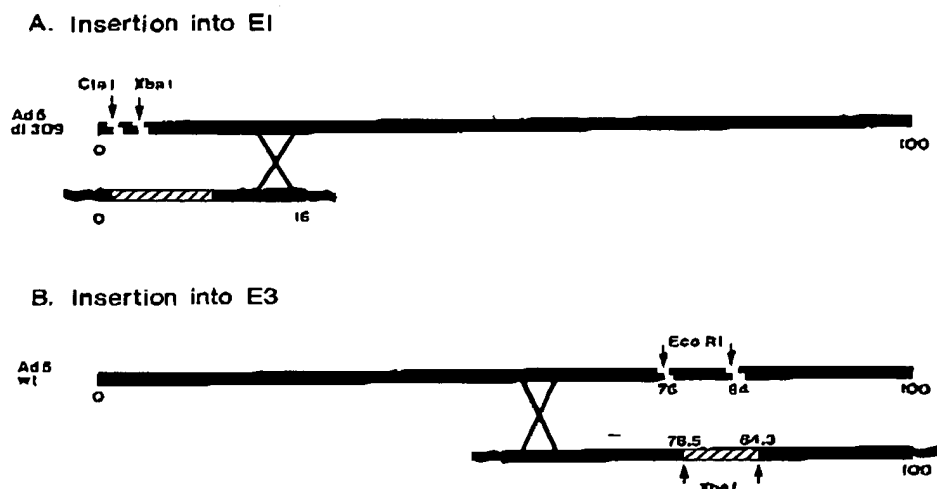


Figure 1. Examples of adenovirus vector construction using conventional approaches. A: Ad5 dl309 DNA is cut with *ClaI* and *XbaI* and mixed with plasmid DNA containing the left end of Ad5 with an insert of foreign DNA (hatched segment). Transfection and in vivo recombination results in rescue of the insert into infectious virus. B: a similar approach for obtaining rescue into E3. Alternative methods involve in vitro ligation of plasmid DNA segments to fragments of virion DNA prior to transfection of 293 cells.

For some purposes the use of such plasmids in the manipulation of the viral genome has a number of advantages over the use of infectious virion DNA. Firstly plasmid DNA is much more economical and easier to prepare than viral DNA, and can be manipulated and analyzed much more rapidly. Secondly the variety of alterations which can be made in viral sequences propagated in bacterial plasmids is much greater than the changes which can be introduced in a molecule whose infectivity must be maintained. An example is illustrated in Fig. 2 which outlines a novel approach for rescue of E1 inserts or mutations into Ad5. We started with pFG140, a circularized form of Ad5 dl309 carrying a 2.2kb DNA insert (pMX2) encoding Ap^r and a bacterial origin of replication and by a series of simple recombinant DNA manipulations converted it to pJM17 (10) which is similar to pFG140 but has a 4.3kb insert. pFG140 is infectious but the pBRX insert in pJM17 exceeds the packaging capacity of the Ad5 capsid. As a result transfection of 293 cells can only give rise to infectious virus following rearrangements which reduce the net size of pJM17 and this

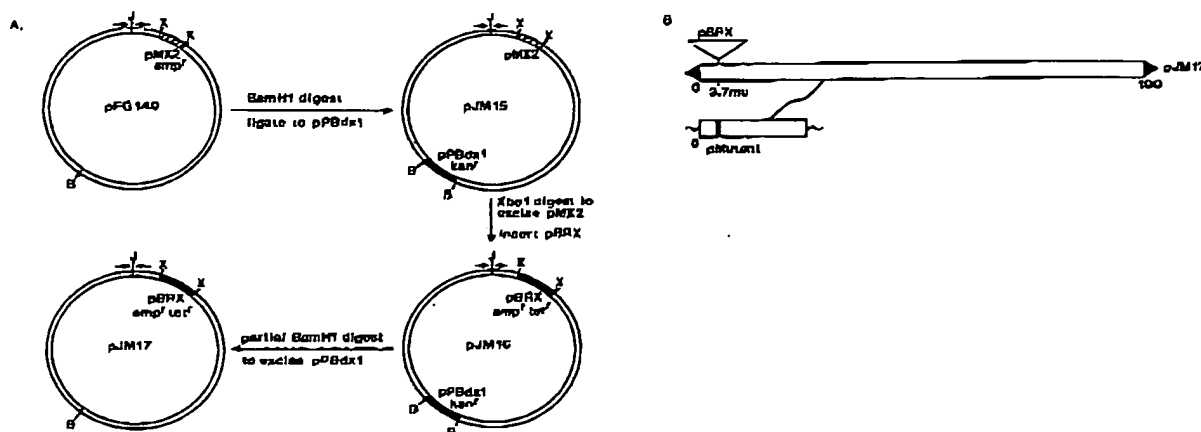


Figure 2. Construction and use of pJM17 for efficient rescue of foreign DNA into E1. Plasmids in B are represented as linear but are actually transfected as intact circular DNA.

happens only very inefficiently. Cotransfection with pJM17 and plasmids containing the left end of Ad5 greatly enhances infectivity and 80-100% of the viral progeny arise from recombination between the cotransfected plasmids (Fig. 2B) providing a simple and efficient method for rescuing E1 mutations and insertions into infectious virus (10).

Fig. 3 illustrates two additional methods for using infectious plasmids to manipulate the Ad5 genome, this time for introducing insertions into E3. In the experiment shown in Fig. 3A we used pFG144 (11), a plasmid derived from a viral vector, dlE1,3, with deletions in E1 and E3 (12), and carrying an insert of pMX2 in the E3 region. Substitution of pMX2 by pPB3 generated a Nm^r plasmid which, following transfection of 293 cells, is able to produce infectious virus encoding resistance to G418. In the experiment shown in Fig. 3B, pPB3 (Nm^r) was substituted with an Ap^r plasmid containing the HSV gB gene linked to the SV40 promoter to generate pGGC161. The latter was used to transfect 293 cells resulting in an $\text{E1}^- \text{E3}^-$ virus, AdgB1, expressing the HSV gB gene product (13). Fig. 3C illustrates yet another approach to rescuing genes into Ad5. In this case we constructed a plasmid with an insertion at an EcoRI site just to the left of E3 in the Ad5 genome. Because this is an essential region of the viral genome this had the effect of rendering the plasmid non infectious. Cotransfection of 293 cells with pFG154neo and a plasmid containing overlapping Ad5 sequences plus an insert of HSVgB resulted in recombination to generate infectious virus containing gB (13).

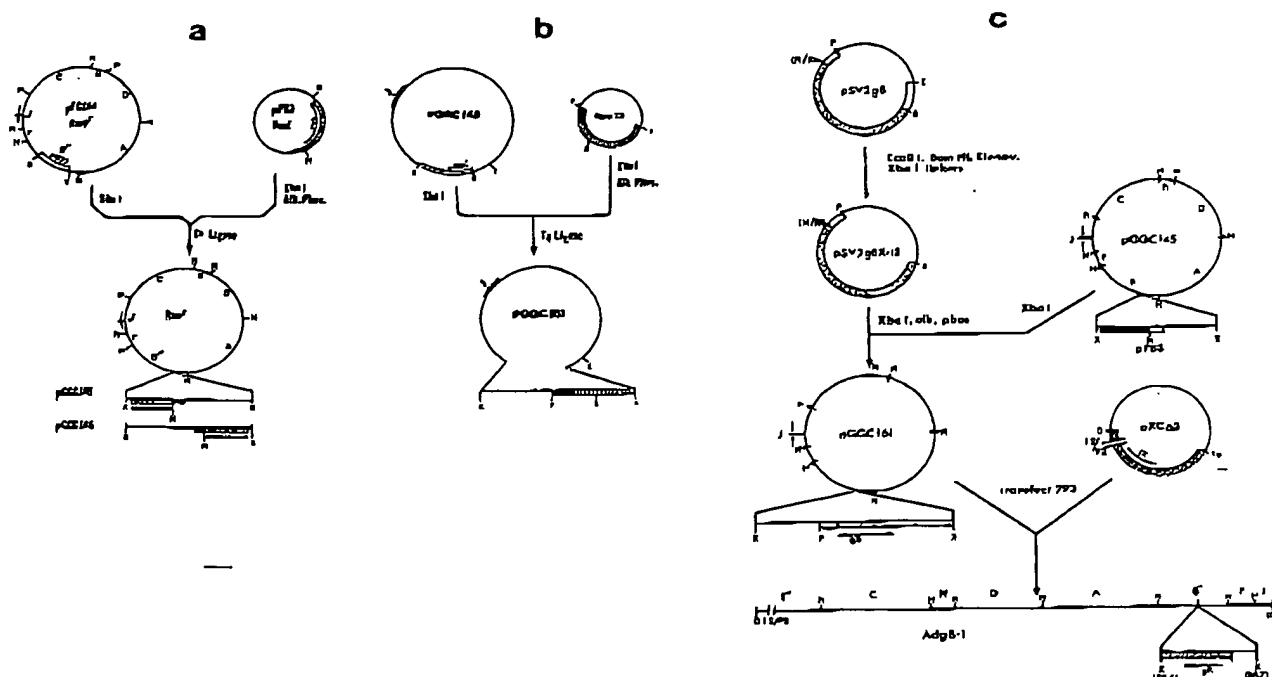


Figure 3. Construction of infectious plasmids containing the gene for neomycin (G418) resistance (A) or HSVlgB (B). The gB coding sequences (hatched segment in pgBX13 and pGGC161) were linked to the SV40 early promoter (solid segment). C: Rescue of the HSVgB gene into E3 of an $E1^+$ virus by cotransfection.

This virus, AdgB2, contains an intact E1 region, and the SV40 early promoter linked to the coding sequences of gB oriented left to right, ie in the same direction as transcription from E3 and the major late promoter. In contrast the SV40-gB chimeric gene in AdgB1 is in the opposite orientation.

Using conventional techniques such as those outlined in Fig. 1, and novel methods as just described, we have constructed and begun to characterize a number of vectors carrying genes encoding various viral glycoproteins. Properties of vectors encoding HSV1 gB are described below.

Expression of gB by AdgB Vectors.

Fig. 4 shows production of gB in AdgB1 infected 293, HeLa, or mouse LTA cells. Because AdgB1 lacks E1 it replicates efficiently only in 293 cells and there is a correspondingly efficient expression of gB in these cells. There also appears to be efficient processing from the nonglycosylated precursor to a species which migrates on SDS-PAGE with a mobility identical to that of the mature protein seen in HSV1 infected cells. In contrast to AdgB1, the $E1^+$ vector, AdgB2, is able to

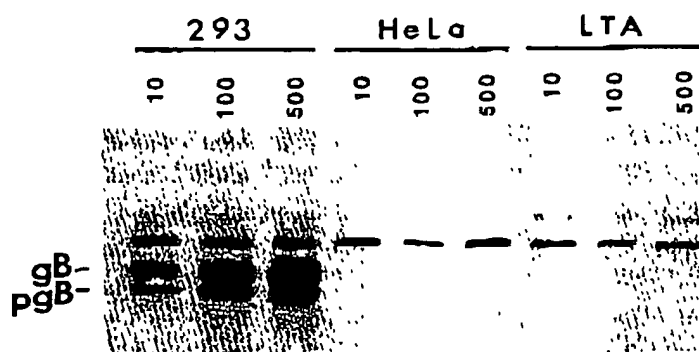


Figure 4.-Expression of gB in cells infected with AdgB1. Infected cells were labelled with ^{35}S -met from 8 to 24hr post infection and cell extracts were immunoprecipitated with a polyclonal anti-gB serum and analyzed by SDS-PAGE.

elicit efficient expression of gB not only in 293 cells but also in human HeLa and R970 cells as well as mouse cells (lines LTA and Z4) at rates comparable to levels of expression in HSV infected cells (Fig.5).

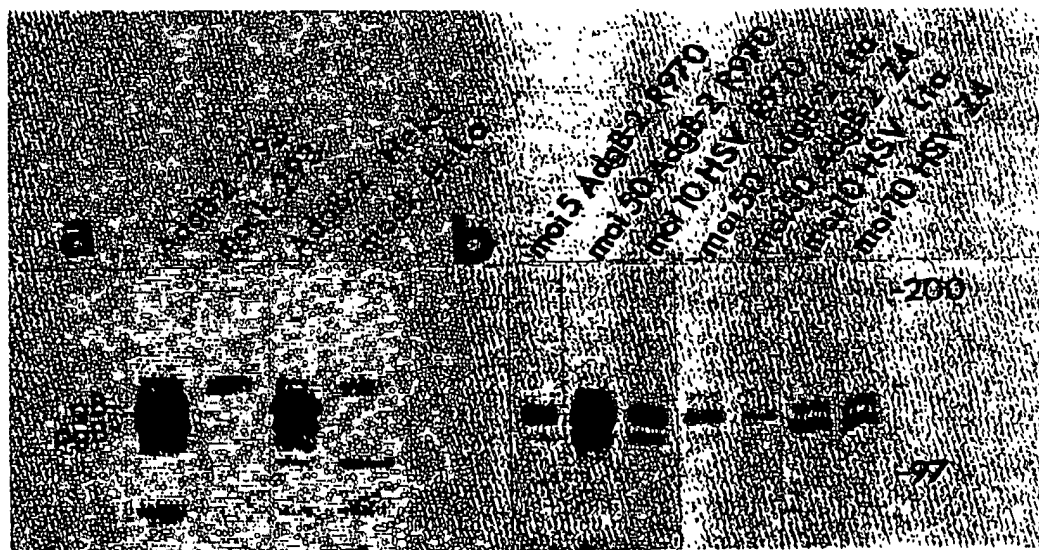


Figure 5. Expression of gB in AdgB2 or HSV1 infected human and mouse cells. Immunoprecipitations were done with a monoclonal anti-gB Ab.

The time course of expression of gB in human R970 cells infected with HSV1 (moi 10) or AdgB1 or 2 (moi 20) is shown in Fig. 6. It is apparent that gB is only synthesized at appreciable levels late in infection with AdgB2 (after 11hrs post infection). In AdgB1 infected cells expression is very inefficient (exposure time for the autoradiogram showing AdgB1 expression was 6 times longer than for the other infections) and is detectable only at very long times post infection.

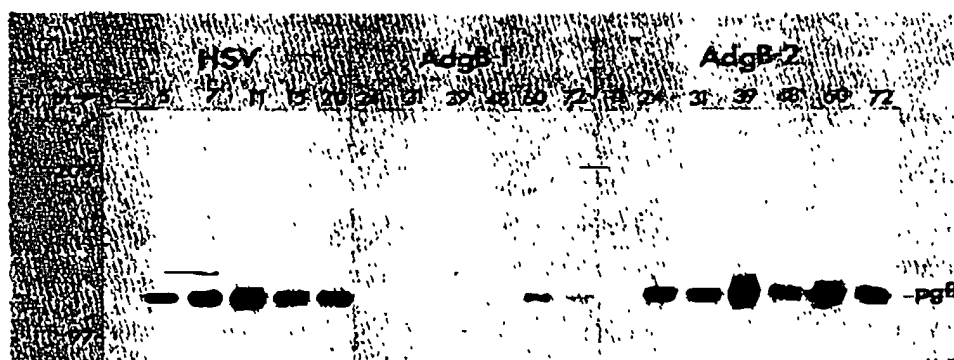


Figure 6. Time course of gB expression in human R970 cells infected with HSV1, AdgB1, or AdgB2.

Because expression of gB in AdgB2 infected cells had kinetics similar to that of late genes, and because it had been reported previously that a promoterless gene inserted into E3 of Ad5 may be expressed from transcripts originating from the major late promoter (7), we carried out S1 and primer extension analyses of mRNA isolated from AdgB2 infected cells. The results of those studies (13) indicated that the SV40 promoter was not responsible for driving transcription of the gB-SV40 chimeric construct to any significant extent but rather that the SV40 sequences may serve as a splice acceptor site for transcripts originating in Ad5 sequences upstream.

Protection Studies.

Although mouse cells are relatively nonpermissive for replication of human adenoviruses the levels of expression of gB in AdgB2 infected mouse cells were nevertheless quite significant. This encouraged us to carry out preliminary studies designed to assess whether immunization of mice with AdgB2 protects against a subsequent challenge with HSV. The results of two experiments (Fig.7) indicate that AdgB2 was

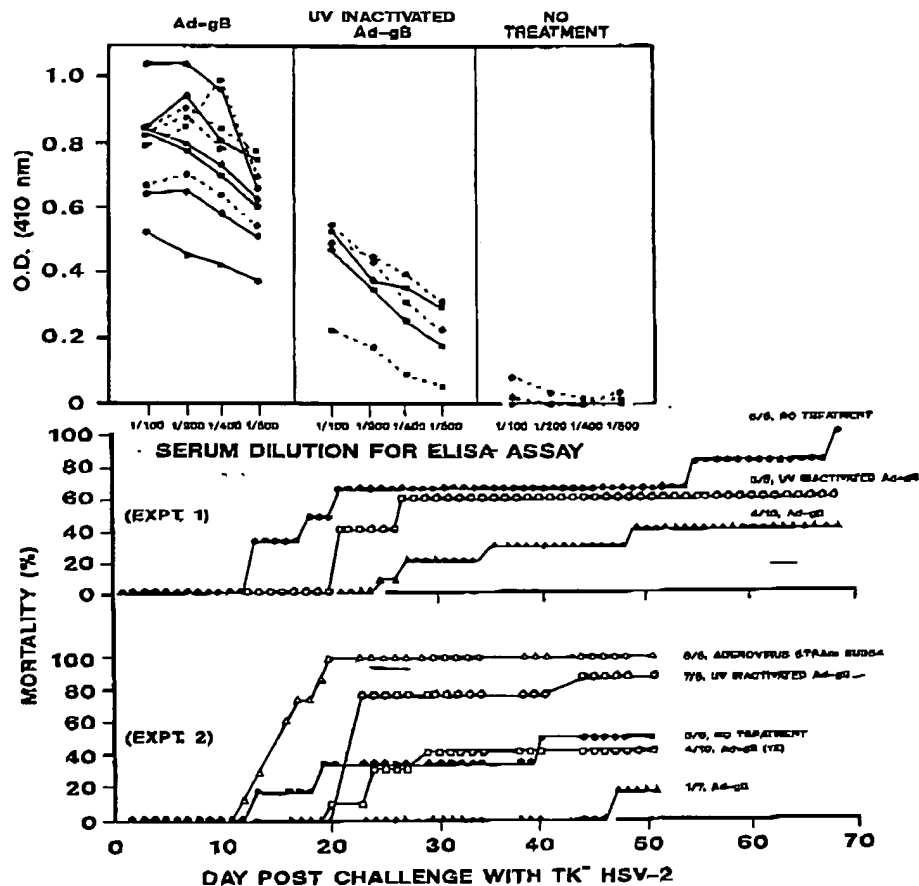


Figure 7. AdgB2 induced protection against HSV2. BALB/cJ mice were inoculated intraperitoneally 3 times at weekly intervals with 5×10^7 pfu of purified AdgB2 or appropriate control viruses and challenged with 10^6 pfu of HSV2 one week after the last adenovirus inoculation. The inset shows the results of assays on anti-gB serum titres for individual mice in the first experiment. Dashed lines represent sera of mice which died within 1-2 months post HSV2 infection and solid lines represent survivors.

able to provide significant protection against killing by HSV2 (HSV2 challenge was used in these experiments because previous studies had determined the appropriate parameters for this serotype in the mouse model. Similar studies are currently underway with HSV1.) In experiment 2 in which mice were exposed to an adenovirus vector which does not carry the gB gene, or to UV inactivated AdgB2, there appeared to be enhanced susceptibility to subsequent challenge with HSV2. The reasons for this effect (if it is indeed a real effect given

the small numbers of animals used in these experiments) are presently unclear.

Just prior to challenge with HSV2, serum samples were obtained from all mice and analyzed for antibody (Ab) titres against HSV1 gB by ELISA (Fig. 7, inset). Two observations are noteworthy from these analyses. Firstly, not only did AdgB2 inoculation induce high levels of Ab against gB in all mice, but UV inactivated AdgB2 was also capable of inducing Ab at levels significantly above background (though lower than with live AdgB2) suggesting that UV treatment did not totally abolish the ability of AdgB2 to express gB in vivo. Secondly there appeared to be no correlation between circulating Ab levels and sensitivity to HSV2 infection.

DISCUSSION

Adenovirus vectors provide an additional tool for the molecular biologist interested in expressing and studying gene products in novel contexts. Depending on the construct, expression can be highly efficient and can occur in cells from a variety of different species, including cell types in which human adenoviruses normally replicate very poorly. Our results as well as a previous report (7) suggest that inserts in E3 can be expressed as a result of transcription driven by the Ad E3 or major late promoter but this may not be a universal phenomenon. Identification of controlling elements which can guarantee high level expression of E3 inserts will require further study and is likely to be a largely empirical process.

From the point of view of development of adenovirus vectors as recombinant vaccines the results of our preliminary in vivo studies are encouraging. A vector expressing VSV G has been used to raise neutralizing Ab against VSV in several different species, and AdgB encoding HSV1 gB was able not only to elicit circulating Ab specific for gB but was able to provide partial protection of mice against a subsequent challenge with HSV.

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GENE 03989

Defective and nondefective adenovirus vectors for expressing foreign genes in vitro and in vivo

(Recombinant virus; major late promoter; gene expression)

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SUMMARY

We have constructed recombinant adenoviruses (Ad), with functional or defective *Ela* genes, which harbor either the hepatitis B (HB) virus *s* gene encoding the HB surface antigen, as well as the pre-S2 epitopes, or the bacterial gene encoding chloramphenicol acetyltransferase (CAT) under control of the Ad major late promoter (MLP). The recombinant viruses defective for *Ela* (Ad.MLP.S2 and Ad.CAT), which can be efficiently propagated only on 293 cells that complement this defect, and the nondefective (Ad.MLP.S2.E1A) recombinant were used to infect a wide spectrum of cells of different origin. The yields of HBs and CAT proteins obtained with these different recombinant viruses demonstrate no real advantage to using nondefective vectors, whatever the cell type infected. The injection into chimpanzees of Ad.MLP.S2 does not elicit the production of antibodies, but can immunologically prime the animals, resulting in a partial protection against HBV challenge.

INTRODUCTION

The human adenovirus (Ad) presents several advantages which can render it a generally useful vector to express foreign genes (Ballay et al., 1985).

In this study, we describe the construction of *Ela*-defective and nondefective recombinant adenoviruses, and compare the efficiencies of expression of the cloned genes in cell culture (i.e., the HBV middle envelope protein gene, including the *s* and the pre-s2 regions of the *s* gene, and the

bacterial reporter gene *cat*). These recombinants make use of the adenovirus major late promoter and a nearly complete copy of the tripartite leader sequence to express the foreign genes (Chow et al., 1977; Lewis et al., 1984). When injected into rabbits, Ad.MLP.S2 elicits the production of antibodies against the middle and major envelope proteins. However, only one out of the two chimpanzees inoculated with this same recombinant was fully protected against hepatitis following a challenge with HBV, while the other experienced a modified HBV infection.

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Abbreviations: Ad, adenovirus; Ag, antigen; anti-HBc, antibodies against HB core Ag; anti-HBs, antibodies against HB surface Ag; bp, base pair(s); CAT, chloramphenicol acetyltransferase; *cat*, gene encoding CAT; *Ela*, gene encoding E1A; *E1b*, gene encoding E1B; HB, hepatitis

B; HBV, HB virus; ID, infectious dose; I.U., international unit(s); kb, kilobase(s) or 1000 bp; MLP, major late promoter; moi, multiplicity of infection; m.u., map unit(s); nt, nucleotide(s); p.i., post infection; P/N, positive/negative; Polk, Klenow (large) fragment of *E. coli* DNA polymerase I; pfu, plaque-forming unit(s); pre-S2, middle envelope protein of HBV; pre-s2, pre-S2-encoding region; RIA, radioimmunoassay; *s*, gene encoding HBsAg; S, major envelope protein of HBV; SV40, simian virus 40; wt, wild type.

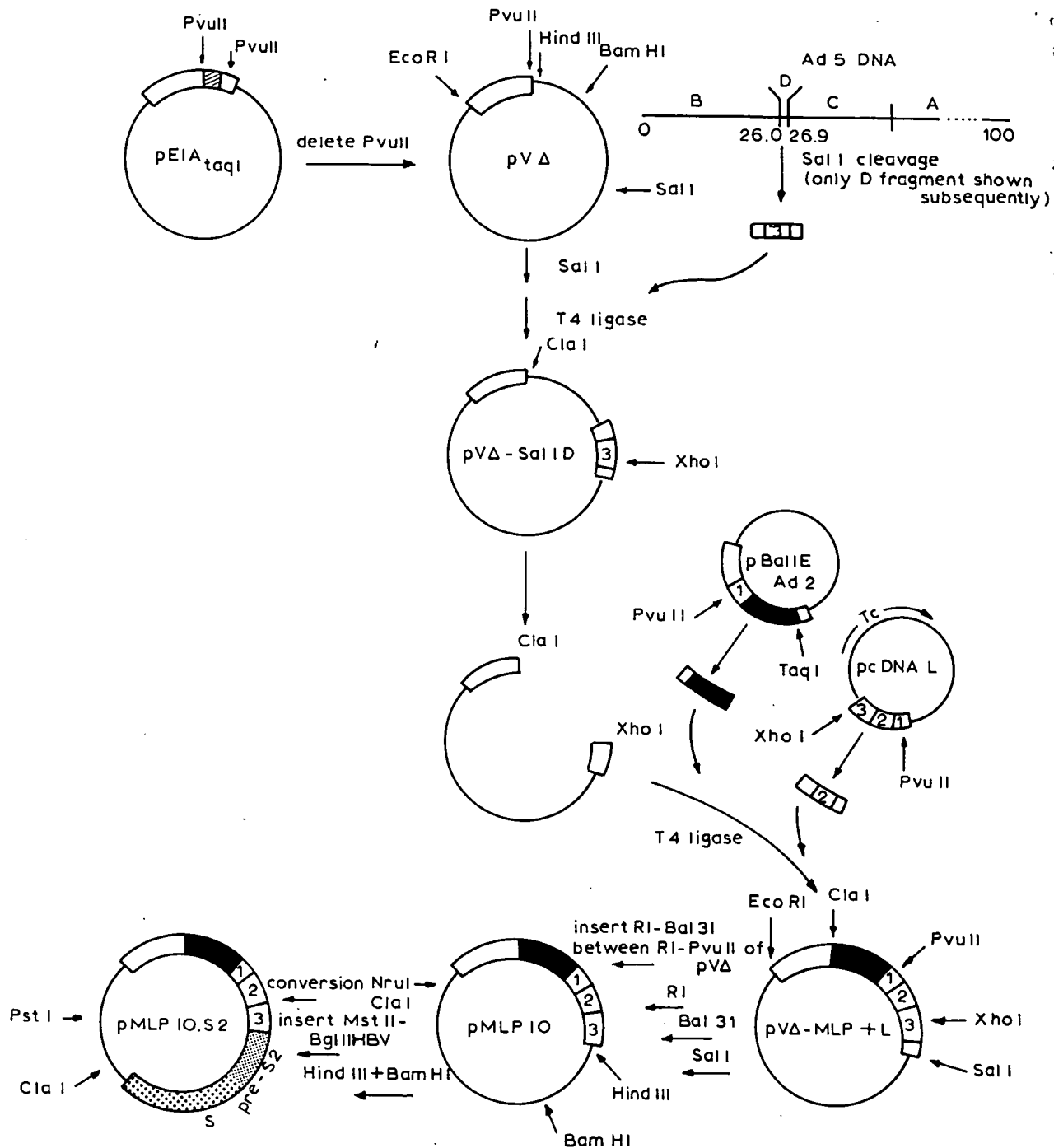


Fig. 1. Construction of plasmid pMLP10.S2. The structure of pEIA_{taqI} has been presented elsewhere (Ballay et al., 1985). Essentially, it retains the leftmost 631 bp of Ad5. Plasmid pVΔ was obtained by deletion of the *Ela* sequences (nt 455-625) using *PvuII*. The adenoviral DNA fragment *SalI*-D (nt 9455-9835), which contains the entire third leader was inserted into the *SalI* site of pVΔ (plasmid pVΔ-SalID). Plasmid pcDNA L, containing the leader sequences derived from an Ad2 cDNA clone, was digested with *XhoI* + *PvuII* while pBalleAd2 (14.7-21.5 m.u.), which contains the MLP, was digested with *TaqI* + *PvuII*. The fragments of interest were then inserted between the *ClaI* and *XhoI* sites of pVΔ-SalID. The resultant plasmid, pVΔ-MLP+L, contains the Ad2 MLP and the intact leader sequences, flanked on their left by the leftmost 455 bp of Ad5 viral genome. This plasmid was linearized by *SalI* and mildly digested with exonuclease BAL 31 to remove the splice donor site located at the end of the third leader. *EcoRI* BAL 31 fragments were inserted between the *EcoRI* and *PvuII* sites of pVΔ. One clone named pMLP10 was found to retain the first and second Ad2 leaders and 80 out of the 90 nt of the third leader. Plasmid pMLP10.S2 was constructed by inserting the fragment *MstII*-*BglII* (nt 3161-1982) of the HBV genome coding for the HBsAg as well as for the pre-S2 epitopes between the *HindIII* and the *BamHI* sites of pMLP10 after filling-in the ends. The *MstII* site precedes the start codon of the pre-s2 region by 9 nt, whereas the *BglII* site is located 64 nt downstream from the poly(A)-addition signal of the s gene. Open boxes, sequences for Ad left end; blackened boxes, MLP from Ad; open boxes marked 1, 2 and 3, Ad leader sequences; stippled boxes, HBV sequence; thin line, plasmid sequence.

RESULTS AND DISCUSSION

(a) Construction of the adenovirus HBV recombinants

The Ad5 recombinants Ad.MLP.S2, with most of the *E1a* region substituted by a chimeric gene, and Ad.MLP.S2.E1A, similar to the former but containing a functional *E1a* gene, were constructed according to the protocol previously described (Ballay et al., 1985). To construct Ad.MLP.S2, a short DNA fragment containing a *Cla*I site was inserted into the *Nru*I site of plasmid pMLP10.S2 (Fig. 1) to allow the ligation of the pMLP10.S2 *Pst*I-*Cla*I fragment to the large *Cla*I fragment of Ad5dl327 DNA (2.6–100 m.u.). In the case of Ad.MLP.S2.E1A, the *Pst*I-*Cla*I fragment of pMLP.S2.E1A (Fig. 2) was directly ligated to the large *Cla*I fragment of Ad5 dl327.

(b) Construction of the adenovirus *cat* recombinants

Recombinant plasmids pMLP.CAT, pMLP.CAT.E1A, and pMLP.CAT.inv. were used to generate corresponding viruses Ad.CAT, Ad.CAT.E1A, and Ad.CAT.inv. (Fig. 3). In Ad.CAT and Ad.CAT.E1A, the DNA segment encoding the CAT protein is positioned downstream from a series of sequences: the Ad5 left terminus (nt 1–455 of Ad5), the Ad2 MLP, a cDNA sequence encoding the Ad2 tripartite leader; it is followed by splicing (nt 4705–4099) and polyadenylation (nt 2770–2553) signals from SV40. Furthermore, the ligation allowed creation of a full copy of the *E1a* gene. In the case of the Ad.CAT.inv., the hybrid transcription unit is also located downstream from the Ad5 left terminus, but in a reverse orientation with respect to viral replication sense.

(c) Growth characteristics and host dependence for viral replication of the adenovirus recombinants

To compare the growth of the Ad.MLP.S2 and Ad.MLP.S2.E1A recombinants in 293 cells and in human cells not transformed by Ad5 (HeLa and HepG2 cells), virus stocks were titrated on these three cell types in a plaque assay. Equivalent titers were found for wt strain and both recombinants on 293 cells. The defective recombinant Ad.MLP.S2 produced no detectable plaques on HeLa cells, and only a small number of plaques on HepG2 cells. Stocks of the Ad.MLP.S2.E1A virus on HeLa or HepG2 cells were comparable to those obtained on 293 cells, thus demonstrating this virus' ability to behave, at least in vitro, as a nondefective, fully competent, wt adenovirus.

With regard to the *cat* recombinant viruses, each one replicates to levels equivalent to Ad5dl327 in 293 cells. However, plating efficiency of Ad.CAT.E1A was identical on 293 and Vero cells, showing the restoration of a functional *E1a* gene for the latter virus.

(d) HBsAg and CAT activity expressed by the recombinant adenoviruses

The infection of 293 and Vero cells by Ad.MLP.S2 at a moi of 10 pfu per cell (as determined by titration on 293 cells) leads to an accumulation in the medium of HBsAg of 6 μ g/10⁶ cells and of 11 μ g/10⁶ cells, respectively (Fig. 4A). HBsAg particles showed a density of 1.2 g/cm³ and carry both middle and major HBV envelope proteins (Fig. 4B).

In view of the usefulness of a eukaryotic vector able to express foreign genes at high levels in a wide spectrum of mammalian cells, we tested our recombinant in several cell lines of different species and embryological origins

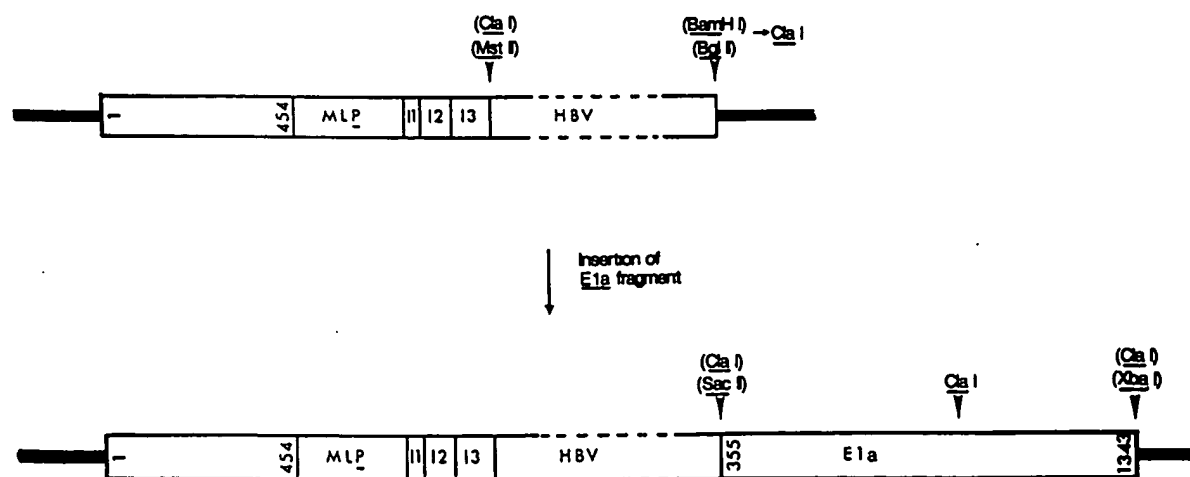


Fig. 2. Construction of plasmid pMLP.S2.E1A. To construct the plasmid pMLP.S2.E1A, the *Cla*I site of pMLP10.S2, created at the end of the HBV sequences by ligation of the *Poll*k-filled ends of *Bgl*II and *Bam*HI, has been used to insert the adenovirus fragment *Sac*II-*Xba*I (nt 353–1340). Thick lines correspond to pBR322 sequences while the cloned nt sequences are boxed. HBV genes, not drawn to scale, are represented as dashed lines.

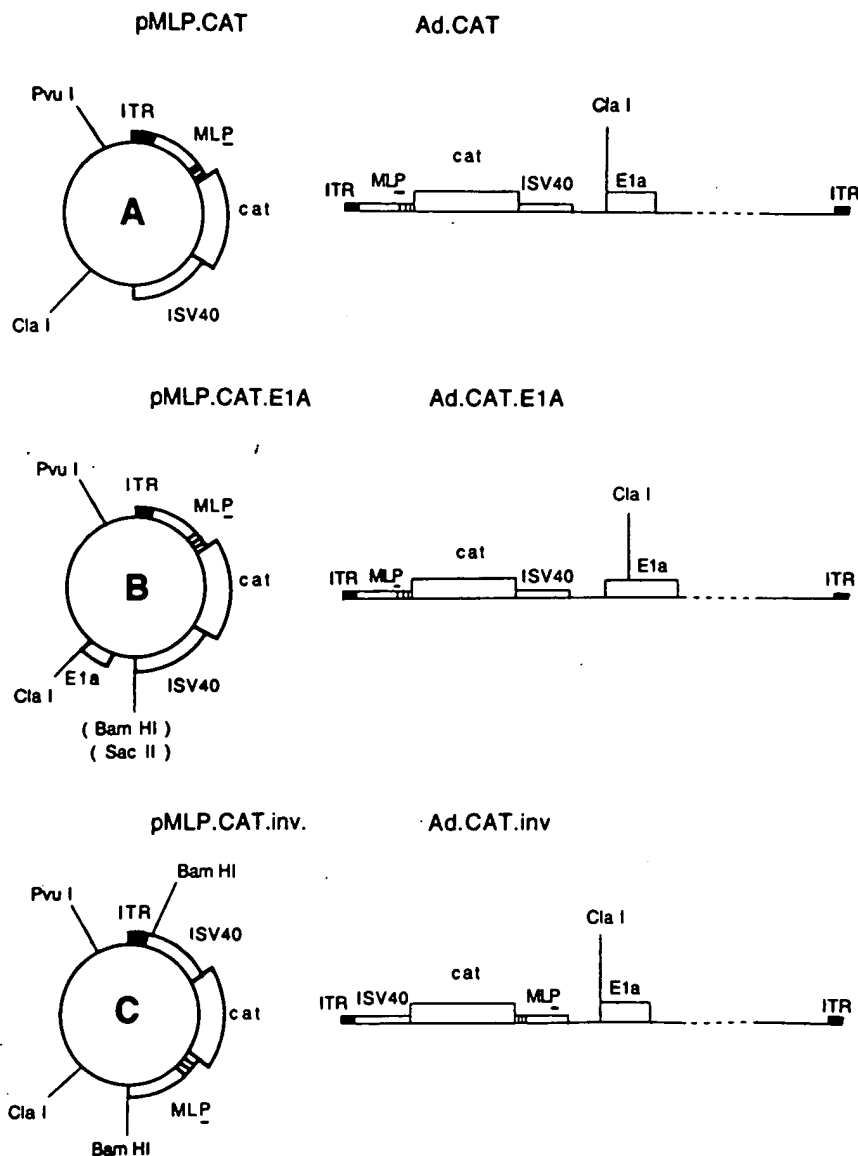


Fig. 3. Construction of Ad.CAT recombinant viruses from plasmids pMLP.CAT (plasmid A), pMLP.CAT.E1A (plasmid B) and pMLP.CAT.inv. (plasmid C). Plasmid pMLP.CAT has been obtained by inserting between the *Hind*III and *Bam*HI sites of pMLP10, the *Hind*III-*Bam*HI fragment from pSV.CAT containing the *cat* region followed by the SV40 intron and polyadenylation sequences. A *Cla*I site was subsequently inserted into the *Nru*I site of pMLP.CAT to construct the recombinant adenovirus following the same way reported for plasmid pMLP10.S2. Plasmid pMLP.CAT.E1A was constructed by insertion of a *Sac*II-*Cla*I fragment, containing nt 353–917 from the left end of Ad5 [except for nt 916, changed to C (underlined), and nt 923 changed to T (underlined)], using the synthetic oligo 5'–GGCAGGTAACATCGATTACCTCCGG, and a mutagenesis kit supplied by Amersham] between the unique *Bam*HI (filled with *Pol*k) and *Cla*I sites of pMLP.CAT. Thus, this plasmid contains the 5'-terminal part of the *E1a* gene, downstream from the *cat* gene and in the same orientation. In regard to plasmid pMLP.CAT.inv., a 117-bp *Sac*II fragment (generated from the *Sac*II sites located at nt 353 and 5794 in the genome of Ad2) was excised from pMLP.CAT and replaced by a synthetic linker *Sac*II-*Bam*HI-*Sac*II. This modified plasmid was fully cleaved with *Bam*HI and then self-ligated to obtain recombinants containing the hybrid transcription unit (Ad.MLP plus tripartite leader plus *cat* gene) in a reverse orientation. Plasmids were digested with *Pvu*II + *Cla*I, ligated to the large *Cla*I fragment of Ad5d1327 and the ligation mixture was used to transfect 293 cells (Graham and Van der Eb, 1973). ITR, inverted terminal repeat of Ad; MLP, major late promoter and tripartite leaders; i SV40, intervening sequence of SV40.

(Table I). The defective Ad.MLP.S2 drove the synthesis of substantial, although variable, amounts of HBsAg in all the cell lines tested. These levels were usually higher than those obtained with the nondefective Ad.MLP.S2.E1A recombinant in all the cell lines but those of lymphoid origin. Under the conditions used in these experiments, the rapid

onset of a lytic infection with the nondefective recombinant may account, at least in part, for this phenomenon. In cells of lymphoid origin, it has been demonstrated that, even in the presence of fully competent wt adenovirus, several steps in the adenovirus replicative cycle are not efficient (Silver and Anderson, 1988). In this context, the combined effects

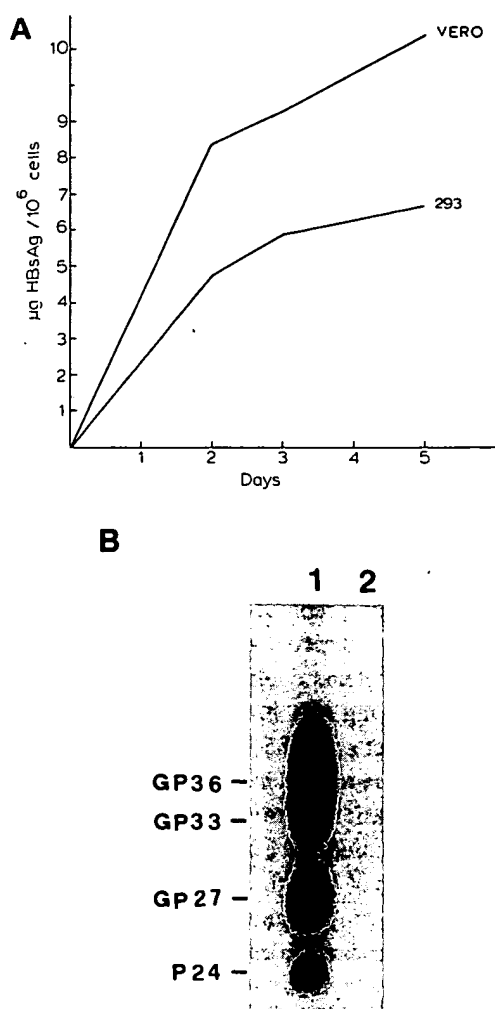


Fig. 4. Adenovirus-mediated HBs production. (Panel A) Cumulative amounts of extracellular HBsAg (expressed in μg) produced after infection of 10^6 293 cells and 10^6 Vero cells by the Ad.MLP.S2 recombinant. HBsAg was detected by RIA (AUSRIA II, Abbott Labs.), and quantified using a parallel line assay with an HBsAg standard (20 ng/ml). (Panel B) Autoradiograph of ^{35}S -labeled polypeptide material from cell-culture medium immunoprecipitated with rabbit anti-HBs antiserum. 48 h after transfection, cells were grown for 1 h in methionine-free medium and radiolabeled for 3 h with $150 \mu\text{Ci/ml}$ of [^{35}S]methionine (1064 Ci/mmol, New England Nuclear) per dish. Medium was clarified by centrifugation for 1 h at $650 \times g$ and concentrated to $50 \mu\text{l}$ using a Centricon 10 micro-concentrator (Amicon). Immunoprecipitation was performed as described previously (Weimer et al., 1987) with all the concentrated medium, and $5 \mu\text{l}$ of antiserum. Immunoprecipitated proteins were analyzed by 0.1% SDS-PAGE following the Laemmli (1970) procedure. After electrophoresis, the gel was soaked for 1 h in a solution of 30% methanol/10% acetic acid, treated with Enhance (New England Nuclear) as indicated by the supplier, dried and exposed to an x-ray film at -70°C . Lanes: 1, 293 cells infected by the Ad.MLP.S2 recombinant; 2, 293 cells infected by wt Ad5. P24 and GP27 represent the 24-kDa nonglycosylated and the 27-kDa glycosylated major envelope proteins. GP33 and GP36 represent the middle envelope proteins glycosylated in the pre-S2 region or in both the pre-S2 and the S regions, respectively.

of the upstream *E1a* enhancer sequence, of the downstream HBV enhancer (which has been reported to work efficiently in lymphoid cells) (Elfassi, 1987), and of the *E1a* gene

TABLE I

Levels of expression of HBsAg after infection with the recombinant adenovirus

Cell lines ^a	Recombinant adenovirus	
	Ad.MLP.S2	Ad.MLP.S2.E1A
Levels of HBsAg (ng/ 10^6 cells) ^b		
Human		
293	6000	3900
HeLa	1540	1150
HepG2	1850	1430
Raji	450	2150
EBV lymphoblastoid	1050	1780
Simian		
Vero	11600	5430
Mouse		
NIH3T3	850	780
L	430	170
Rabbit		
RK13	950	860
537	480	250

^a 293 (Harrison et al., 1977); HeLa (Gey et al., 1952); HepG2 (Knowles et al., 1980); Raji (Pulvertaft, 1964); NIH3T3 (Jainchill et al., 1969); L (Sanford et al., 1948); RK13 (Beale et al., 1963).

^b Cells were infected at an moi of 10 pfu per cell; supernatants were collected 120 h p.i. and tested for HBsAg reactivity as described (Ballay et al., 1985).

product, contribute to the high rate of transcription from the Ad MLP. The level of HBsAg and of *cat* expression observed in Vero cells and in other non-Ad5-transformed cells is very high despite the fact that the Ad MLP is known to be tightly controlled throughout the viral infectious cycle. However, it has been demonstrated that by culturing Ad-infected HeLa cells for prolonged periods in the presence of an inhibitor of viral replication, not only is there an important production of *E1a* and *E1b* early gene mRNAs along with their corresponding proteins, but the late viral protein hexon, is also accumulated (Gaynor et al., 1982).

Summarized in Fig. 5A are the results dealing with the intracellular accumulation of CAT, upon infection of either 293 or Vero cells by Ad.CAT, which confirm those obtained with the HBV recombinants. In 293 cells, maximal accumulation occurs 48 h p.i. Then, a rapid decrease in CAT activity takes place, correlated with the strong cytopathic effects observed (Fig. 5A). Interestingly, the presence of a viral copy of the *E1a* gene does not alter *cat* expression. Neither is there a difference in CAT levels with Ad.CAT and Ad.CAT.E1A in 293-infected cells, or a difference between 293 and Vero cells infected with Ad.CAT.E1A. However, a displacement in the activity curve is observed with

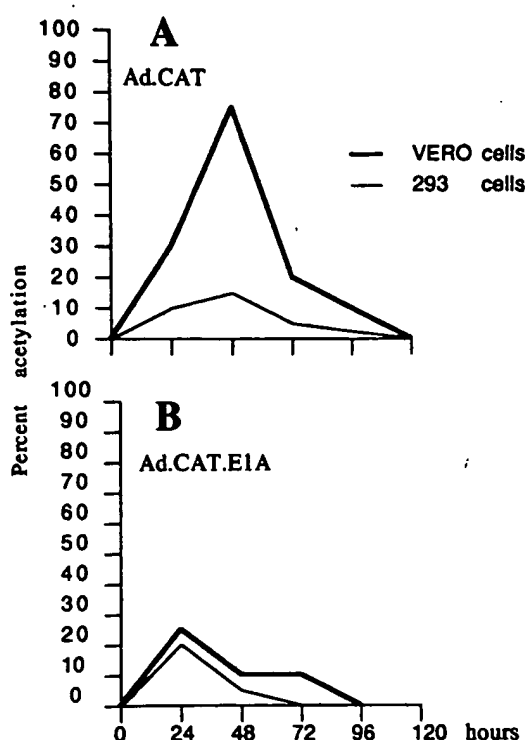


Fig. 5. Kinetics of CAT accumulation following infection with either Ad.CAT (A) or Ad.CAT.E1A (B). The 293 and Vero cells were infected at an moi of 10 pfu/cell and harvested every 24 h. CAT transient assays were performed as described by Gorman et al. (1982). The % acetylation is given for 0.5 μ g of total proteins being tested.

Ad.CAT.E1A (Fig. 5B). The maximum CAT level occurs 24 h p.i. instead of 48 h. We propose, as an explanation, that *cis*-acting functions are brought by the *E1a* gene and facilitate the transition from the early to the late step of viral replication. Alternatively, the E1A could be synthesized at higher levels when the *E1a* gene is brought by the viral genome and would thus facilitate this transition.

To investigate the role of the *E1a* enhancer sequence in our recombinants, we have constructed the Ad.CAT.inv. defective virus, containing the cat hybrid transcription unit in a reverse orientation with regard to the *E1a* enhancer sequence. Maximum CAT levels are summarized in Table II. There is no significant difference between Ad.CAT or Ad.CAT.E1A, and Ad.CAT.inv. in 293 cells, compared to Vero cells where the maximum CAT level is about ten to 100 times higher when the *E1a* enhancer is adjacent to the MLP.

Irrespective of the mechanism involved, our results indicate that adenovirus can be a generally useful vector to express foreign genes at high levels in a wide spectrum of cells.

(c) Biological activity of Ad.MLP.S2 in rabbits and chimpanzees

To test the ability of the adenovirus recombinant Ad.MLP.S2 to direct the synthesis in vivo of HBsAg par-

TABLE II

Comparison of maximum CAT accumulation levels following infection with recombinant adenovirus, in 293 cells and Vero cells

Virus ^a	Cell lines ^b	
	293	Vero
Ad.CAT	17	77
Ad.CAT.E1A	20	23
Ad.CAT.inv	20	<5

^a Infections were performed at an moi of 10 pfu per cell in every case. Values correspond to the % of acetylation, calculated as described and corrected for 0.5 μ g of total proteins.

^b For references, see Table I, footnote a.

ticles carrying pre-S2 epitopes, rabbits were inoculated i.v. with highly purified preparations of either recombinant or wt Ad5. Antibodies directed against both middle and major HBV envelope proteins could easily be detected as of the first week following the injection, as we have already reported (Ballay et al., 1985; data not shown).

The ability of the recombinant Ad.MLP.S2 virus to protect against hepatitis was then tested in two chimpanzees which were vaccinated twice with a three-month interval by i.v. administration of 10^9 pfu of the recombinant per animal (Fig. 6). Neither of the animals developed side effects during the vaccination period. All sera were negative for elevations in liver enzyme activity and for evidence of exposure to HBV antigens. The control chimpanzee 2026 (Fig. 6) that had not received the adeno-HBV recombinant, developed a typical B-type hepatitis. HBsAg appeared eleven weeks after the HBV challenge and reached a P/N ratio value of 43. Anti-HBc and biochemical evidence of hepatitis were detected after 18 and 20 weeks, respectively. HBsAg levels declined and disappeared 30 weeks after challenge, coincident to the appearance of anti-HBs antibodies. Even in the absence of circulating anti-HBs, the chimpanzee Oscar was immunologically primed and the rapid and sustained antibody response after challenge with live HBV is in keeping with this hypothesis. The same phenomenon of protection after challenge even in the absence of seroconversion to anti-HBs was also described by Moss et al. (1984) in chimpanzees vaccinated by a recombinant vaccinia virus.

The chimpanzee Oscar, which received the recombinant adenovirus, showed no detectable HBsAg or biochemical evidence of hepatitis. Six weeks after the challenge, anti-HBs appeared and reached high levels that persisted during the rest of the experiment. On the contrary, the second animal, Theo, developed a mild case of hepatitis characterized by the appearance of HBsAg 14 weeks after the challenge, followed by a slight elevation of alanine amino-

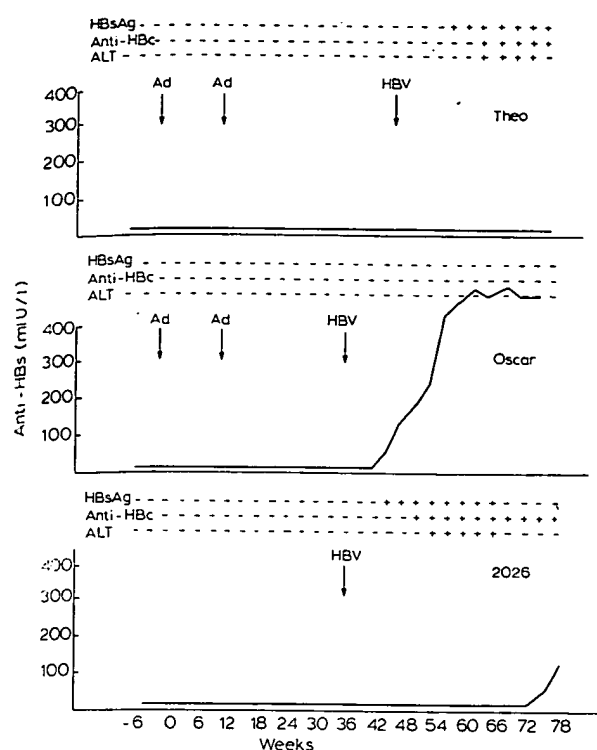


Fig. 6. Chimpanzee vaccination. Three chimpanzees, sero-negative for HBV markers, as determined with commercially available RIA (HBsAg; AUSRIA; anti-HBs; AUSAB; anti-HBc; CORAB; antiHBc IgM; CORZYME M; Abbott Laboratories), were used. None of the animals showed evidence of hepatitis as judged by normal levels of alanine aminotransferase and aspartate aminotransferase. In the animals named Oscar and Theo, 1 ml containing 10^9 pfu of the recombinant virus was injected and a booster was performed twelve weeks later. The recombinant adenovirus stock was free of any contamination by HBsAg particles as judged by RIA and electron microscopy. The third chimpanzee, numbered 2026, did not receive the vaccine, and served as a control. The three chimpanzees were subsequently challenged i.v. with $10^{3.5}$ chimpanzee ID_{50} units of live HBV (subtype ayw, strain MS-2). Oscar and Theo received HBV, 24 weeks and 36 weeks, respectively, after the booster inoculation. Such a dose and strain have been demonstrated to consistently induce HB in susceptible chimpanzees (Barker et al., 1975). Serum samples were obtained at weekly intervals after the HBV challenge. Alanine aminotransferase (ALT) activity is expressed in I.U./liter and levels exceeding twice the mean of the baseline are considered high and indicated by the symbol +. Anti-HBs antibodies are expressed as the ratio of sample c.p.m. : negative control c.p.m. (S/N). Positive HBsAg values (2.1 P/N) and positivity for anti-HBc antibodies are indicated by the symbol +.

transferase levels. Thus, the chimpanzee Theo developed both serological evidence of HBV infection and biochemical evidence of liver disease in spite of an anterior inoculation with the recombinant adenovirus. Similar results, i.e., full protection of one animal and modified HBV-induced disease in another, have recently been obtained using an oral HB vaccine based on a live recombinant adenovirus (Lubeck et al., 1989).

Although additional information is needed concerning the efficacy to induce a protective immune response in

animals, adenovirus constitutes a useful model towards the development of live vaccines. In this regard, a vaccine containing live infectious adenovirus in an enteric-coated dosage form is already marketed, and has proven to be effective and at the same time free of significant side effects (Chanock et al., 1966; Edmonson et al., 1966). Moreover, the release of adenovirus into the intestine is followed by its replication there without causing adenoviral disease, yet inducing the formation of adenovirus antibodies that render adenoviral recombinants suitable candidates for the development of vaccines against enteric infections.

(f) Conclusions

(1) No real advantage in using nondefective vectors for the production in cell cultures of foreign proteins can be demonstrated.

(2) The HBsAg synthesized by the recombinant adenovirus Ad.MLP.S2 is similar, if not identical, to material from the serum of human HBV carriers. The HBsAg was excreted as 22-nm particles carrying pre-S2-encoded determinants.

(3) The preliminary results from chimpanzees receiving i.v. the Ad.MLP.S2 recombinant show that only one out of two injected animals was protected against a challenge with HBV, the second animal being insufficiently protected by the vaccination. The injection of chimpanzees with HB vaccines generally constitutes a safety test, however, the potential of our adenovirus recombinant as a live vaccine is being assessed by oral administration since this would be the preferred route of vaccination.

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L2	7851 S L1 AND PROMOTER#
L3	1286 S L2 AND (CMV OR CYTOMEGALOVIRUS)
L4	1148 S L3 AND EXPRESS?
L5	599 DUP REM L4 (549 DUPLICATES REMOVED)
L6	688 DUP REM L3 (598 DUPLICATES REMOVED)
L7	96 S L6 NOT L5 E CRYSTAL RONALD/AU
L8	465 S E5
L9	70 S L8 AND ANTITRYPSIN
L10	64 DUP REM L9 (6 DUPLICATES REMOVED)
L11	236 S L8 AND ADENOVIR?
L12	9 S L11 AND CMV
L13	6 DUP REM L12 (3 DUPLICATES REMOVED)
L14	14 S L11 AND ANTITRYPSIN
L15	10 DUP REM L14 (4 DUPLICATES REMOVED)
L16	21 S L9 AND LUNG
L17	21 DUP REM L16 (0 DUPLICATES REMOVED)

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Adenovirus-Mediated Transfer of a Recombinant α 1-Antitrypsin Gene to the Lung Epithelium in Vivo

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The respiratory epithelium is a potential site for somatic gene therapy for the common hereditary disorders α 1-antitrypsin (α 1AT) deficiency and cystic fibrosis. A replication-deficient adenoviral vector (Ad- α 1AT) containing an adenovirus major late promoter and a recombinant human α 1AT gene was used to infect epithelial cells of the cotton rat respiratory tract in vitro and in vivo. Freshly isolated tracheobronchial epithelial cells infected with Ad- α 1AT contained human α 1AT messenger RNA transcripts and synthesized and secreted human α 1AT. After in vivo intratracheal administration of Ad- α 1AT to these rats, human α 1AT messenger RNA was observed in the respiratory epithelium, human α 1AT was synthesized and secreted by lung tissue, and human α 1AT was detected in the epithelial lining fluid for at least 1 week.

ONE OF THE HURDLES TO OVERCOME in most forms of somatic gene therapy is the specific delivery of the therapeutic gene to the organs manifesting the disease. The lung presents special advantages because a functional gene can be delivered directly to the respiratory epithelium by means of tracheal instillation. The disadvantage of such an approach is due to the normal biology of the respiratory epithelium; only a small proportion of alveolar and

airway epithelial cells go through the proliferative cycle in 1 day, and a large proportion of the cells are terminally differentiated and are, therefore, incapable of proliferation (1). In this regard, it may be difficult to transfer functional genes to the respiratory epithelium by means of vectors (such as retroviruses) that require proliferation of the target cells for expression of the newly transferred gene (2).

To circumvent the slow target-cell proliferation, we have used a recombinant adenoviral vector to transfer a recombinant human gene to the respiratory epithelium in vivo. Host cell proliferation is not required for expression of adenoviral proteins (3, 4), and adenoviruses are normally tropic for the respiratory epithelium (5). Other advantages of adenoviruses as potential vectors for human gene therapy are as follows: (i) recombination is rare; (ii) there are no known associations of human malignancies with

adenoviral infections despite common human infection with adenoviruses; (iii) the adenovirus genome (which is a linear, double-stranded piece of DNA) can be manipulated to accommodate foreign genes of up to 7.0 to 7.5 kb in length; and (iv) live adenovirus has been safely used as a human vaccine (3-8).

The adenovirus (Ad) major late promoter (MLP) was linked to a recombinant human α 1AT gene (9) and was incorporated into a replication-deficient recombinant (Fig. 1) (5, 10). The vector has a deletion of a portion of the E3 region (that permits encapsidation of the recombinant genome containing the exogenous gene) and a portion of the viral E1a coding sequence (that impairs viral replication) but contains an insert of an α 1AT expression cassette (Fig. 1) (10, 11). After packaging into an infectious, but replication-deficient virus, Ad- α 1AT is capable of directing the synthesis of human α 1AT in Chinese hamster ovary (CHO) and human cervical carcinoma (HeLa) cell lines (10).

We obtained tracheobronchial epithelial cells by brushing the epithelial surface of the tracheobronchial tree from the lungs of the cotton rat [*Sigmodon hispidus*, an experimental animal used to evaluate the pathogenesis of respiratory tract infections caused by human adenoviruses (12)]. The freshly removed cells infected in vitro with Ad- α 1AT expressed human α 1AT mRNA transcripts, as demonstrated by in situ hybridization with a ³⁵S-labeled antisense human α 1AT RNA probe (Fig. 2). In contrast, no human α 1AT mRNA transcripts were observed in uninfected, freshly isolated tracheobronchial epithelial cells. Human α 1AT mRNA transcripts in the infected cells were capable of directing the synthesis and secretion of human α 1AT, as shown by biosynthetic labeling and immunoprecipitation with a specific antibody to human α 1AT (Fig. 2E). The newly synthesized, secreted α 1AT was human α 1AT, as shown by the fact that human α 1AT (Fig. 2E, lane 3), but not cotton rat serum, blocked the antibody to human α 1AT.

Ad- α 1AT transferred the recombinant α 1AT gene to the cotton rat lung in vivo (Fig. 3). Human α 1AT transcripts were observed in the lungs 2 days after intratracheal instillation of Ad- α 1AT, but not in lungs of animals that received only phosphate-buffered saline (PBS) or in lungs of animals that received the Ad5 E1a-deletion mutant, Ad-dl312 (13). Biosynthetic labeling and immunoprecipitation of extracellular protein from lung fragments removed from infected animals demonstrated that de novo synthesis and secretion of human α 1AT also occurred (Fig. 3B, lanes 11

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through 15); this was not observed in uninfected animals (lane 10) or in animals infected with Ad-dl312. The de novo expression of the human α 1AT protein lasted at least 1 week (lane 15), and the secreted human α 1AT was functional, as shown by its ability

to form a complex with its natural target, human neutrophil elastase (NE) (lanes 16 through 18).

Two lines of evidence demonstrated that the infection of the cotton rat lung with Ad- α 1AT took place in vivo and was not the

result of virus carried over into the in vitro biosynthetic analysis. First, immediate evaluation of lung tissue removed 2 and 4 days after in vivo infection with Ad- α 1AT revealed human α 1AT mRNA transcripts in the respiratory epithelial lining fluid of cotton rats 3 days after infection with Ad- α 1AT, showed no evidence of infectious virus, and was capable of directing the biosynthesis of human α 1AT, as evidenced by exposure of the cell line to epithelial lining fluid and incorporation of labeled methionine, followed by immunoprecipitation analysis in a manner identical to that used for the analysis of the α 1AT biosynthesis by the lung fragments.

Evaluation of the cotton rat lung by *in situ* hybridization with antisense and sense human α 1AT RNA probes revealed human α 1AT mRNA transcripts in lung cells of animals infected with Ad- α 1AT, but not in those of uninfected animals (Fig. 4). The expression of human α 1AT mRNA transcripts was patchy, as could be expected from the method of intratracheal administration of Ad- α 1AT; more uniform expression should be achievable by modifications of vector delivery methods, such as by aerosol. Consistent with the observation that cotton rat respiratory epithelial cells were easily infected *in vitro* (Fig. 2), most of the transcripts were in epithelial cells; the available methodology do not permit an accurate assessment of the distribution of expression among the multitude of epithelial cell types in the lung. Occasional grains were observed within interstitial cells.

Evaluation of the fluid lining the epithelial surface of the lungs with a human α 1AT-specific enzyme-linked immunosorbent assay (ELISA) demonstrated the presence of human α 1AT in animals infected with Ad- α 1AT, but not in those infected with the deletion mutant virus Ad-dl312 in uninfected animals (Fig. 5). Human α 1AT could be detected at all the periods evaluated (days 1 to 7 after Ad- α 1AT infection). No adverse effects were observed in the animals at any time after infection with either Ad-dl312 or Ad- α 1AT. Because the methods available for administration of α 1AT to the animals result in variable delivery and retention of the vector, it is difficult to make quantitative animal-to-animal comparisons. Thus, the time course for α 1AT expression cannot be accurately determined at this time, although the de novo biosynthesis data demonstrate that the lung is actively synthesizing human α 1AT at day 7 (Fig. 3B, lane 15).

Our findings are relevant to gene therapy strategies for human diseases. The two common lethal hereditary disorders of humans, α 1AT deficiency (allelic frequency

Fig. 1. Recombinant Ad vector. (Top) Wild-type Ad type 5 (Ad5) genome showing the E1a, E1b [map units (mu) 1.3 to 11.2; 100 mu = 36 kb], and E3 (mu 76.6 to 86.0) regions. The recombinant vector Ad- α 1AT is constructed by deleting the majority of the E3 region and 2.6 mu from the left end of Ad5 and adding to the left end the α 1AT expression cassette from the plasmid pMLP- α 1AT, which contains regulatory sequences and a recombinant human α 1AT gene (10). (Bottom) Details of the α 1AT expression cassette. ITR, inverted terminal repeat. To construct the recombinant viral vector Ad- α 1AT, we ligated the expression cassette with C1a I-precut Ad-dl327 DNA (23) (to remove a portion of the E1a region from Ad-dl327). The recombinant adenovirus DNA was transfected into the 293 cell line (24, 25), where it was replicated, encapsidated into an infectious virus, and isolated by plaque purification. Individual plaques were amplified by propagation in 293 cells, and the viral DNA was extracted (26). The intactness of the DNA of the recombinant virus was confirmed before use by restriction fragment analysis and Southern (DNA) blot. Stocks of Ad- α 1AT and the Ad5 E1a deletion mutant Ad-dl312 were propagated and titered in 293 cells (24). The virus was released from infected cells 36 hours after infection by five cycles of freeze-thawing. For some *in vivo* experiments Ad- α 1AT was further purified with CsCl (25).

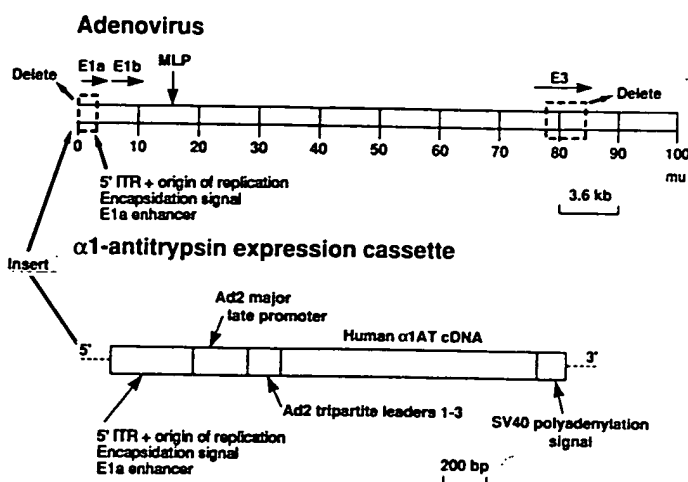


Fig. 2. Expression of human α 1AT in respiratory epithelial cells freshly isolated from cotton rats infected with Ad- α 1AT *in vitro*. (A) Ad- α 1AT-infected cells, antisense probe. (B) As in (A) but for uninfected cells. (C) Ad- α 1AT-infected cells, sense probe. (D) As in (C) but for uninfected cells. (E) Human α 1AT biosynthesis and secretion. We anesthetized cotton rats (methoxyflurane inhalation), exposed the trachea and lungs through a midline thoracic incision, and perfused the pulmonary vasculature with LHC-8 medium (Biofluids) to remove blood. The trachea was transected, and the tracheobronchial epithelial cells (to the second order bronchi) were recovered with a cytologic brush. The epithelial cells were gently pelleted (300g, 8 min, 23°C), resuspended in LHC-8 medium, plated on fibronectin-collagen-coated plates (27), and infected with 2×10^7 plaque-forming units (PFU) of Ad- α 1AT in LHC-8 medium or, as a control, exposed only to LHC-8 medium. After 1 day, we evaluated expression of α 1AT mRNA transcripts in cytocentrifuge preparations by the technique of *in situ* hybridization (28, 29) with 35 S-labeled sense and antisense RNA probes (1.2×10^5 cpm/ μ l) prepared in pGEM-3Z (Promega). After hybridization, the cells were exposed to autoradiography film for 1 week and counterstained with hematoxylin and eosin (HE; all panels $\times 520$). In (E), the cells were infected as in (A). After 1 day, the cells were labeled with [35 S]methionine (500 μ Ci/ml, 24 hours, 37°C), and the supernatant was evaluated by immunoprecipitation with goat antibodies to human α 1AT (Cappel), SDS-polyacrylamide gel electrophoresis, and autoradiography (30). Lane 1, uninfected cells; lane 2, Ad- α 1AT-infected cells; and lane 3, Ad- α 1AT-infected cells with unlabeled human α 1AT added to block the antibody. The position of migration of the 52-kD human α 1AT is indicated by the arrow.

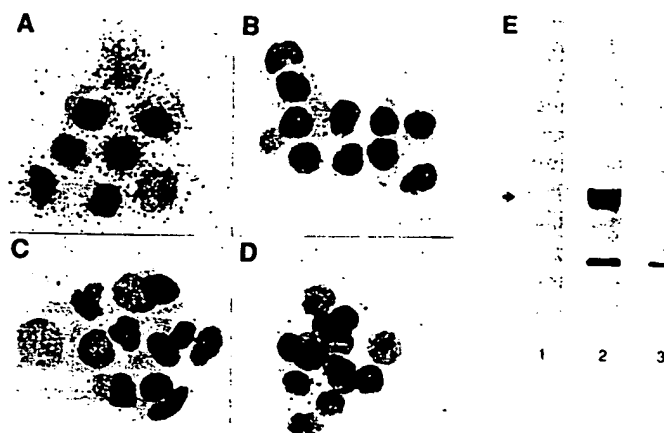
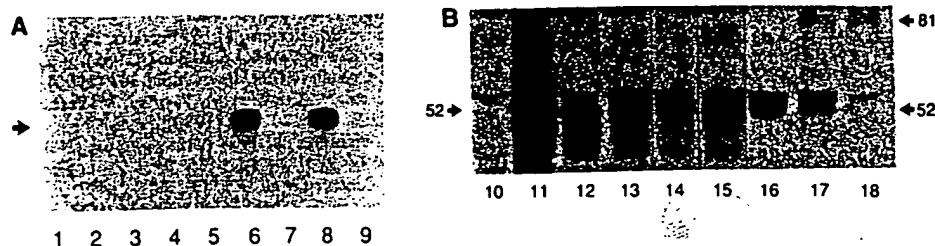


Fig. 3. Expression of human α 1AT mRNA transcripts and synthesis and secretion of human α 1AT by cotton rat lung after Ad- α 1AT infection in vivo. Cotton rats were anesthetized, and Ad- α 1AT was diluted in 300 μ l of PBS to 10^8 PFU/ml and instilled into the trachea. Controls included 300 μ l of PBS or 300 μ l of PBS with Ad-dl312 at 10^8 PFU/ml. After 1 to 7 days, lungs were exposed and lavaged, and the pulmonary vasculature was perfused with methionine-free LHC-8 medium. (A) We extracted total RNA (11), treated the RNA with an excess of deoxyri-

bionuclease (DNase) (RQ1 RNase-Free DNase, Promega), converted the RNA to cDNA by standard techniques, and amplified the cDNA by the polymerase chain reaction (PCR) for 25 cycles (32) with an adenoviral-specific primer in the tripartite leader sequences (Fig. 1) and a human α 1AT exon III-specific antisense primer (33). PCR products were evaluated by agarose gel electrophoresis, followed by Southern hybridization with a human α 1AT cDNA probe that was 32 P-labeled by random priming (34). The size of the expected fragment (1062 bp) is indicated by the arrow. Reverse transcriptase was present in lanes, 2, 4, 6, and 8. Lanes 1 and 2, uninfected lung RNA from the cotton rat (PBS control); lanes 3 and 4, 2 days after infection with Ad-dl312. Lanes 5 and 6, cotton rat 2 days after infection with Ad- α 1AT; lanes 7 and 8, a different rat, treated as in 5 and 6; lane 9, PCR control without RNA or DNA template. (B) At various times after infection, the lungs were minced, incubated for 1 hour in methionine-free LHC-8 medium (37°C), and then incubated for 24 hours in medium with [35 S]-methionine (1 ml of medium/150 mg of tissue; 500 μ Ci/ml). The supernatant was then evaluated by immunoprecipitation with a rabbit antibody to human α 1AT (Boehringer Mannheim), SDS-polyacrylamide



gels, and autoradiography as in Fig. 2E. Trichloroacetic acid-precipitable radioactivity was evaluated by immunoprecipitation (for lanes 10 to 15, 2×10^6 cpm; for lanes 16 to 18, 1×10^6 cpm). Autoradiogram exposures for lanes 10 to 15 were identical; lanes 16 to 18 were evaluated at a different time, and the exposures adjusted such that the intensity of the α 1AT band without NE was similar to that in lane 13. We evaluated the ability of the synthesized human α 1AT to inhibit its natural substrate, NE, by incubating the supernatant with various dilutions of active NE (30 min, 23°C) before immunoprecipitation. Lane 10, uninfected control; lane 11, 1 day after infection with Ad- α 1AT; lane 12, same as in 11 but with antibody exposed to unlabeled human α 1AT before immunoprecipitation; lanes 13 through 15, 2, 3, and 7 days, respectively, after infection with Ad- α 1AT; lanes 16 through 18, 2 days after infection with Ad- α 1AT and with 3 nM, 30 nM, and 300 nM NE added to the supernatant before immunoprecipitation, respectively. The uninfected control evaluated in parallel to lanes 16 through 18 demonstrated no complexes. Indicated is the size of human α 1AT (52 kD) and the human α 1AT-human NE complex (81 kD).

0.01 to 0.02) and cystic fibrosis (CF; allelic frequency 0.022), have their major clinical manifestations in the lung (9, 14). In α 1AT deficiency, mutations of coding exons of the 12.2-kb α 1AT gene result in decreased serum and, hence, lung levels of α 1AT, an antiprotease that normally protects the lung from destruction by the powerful proteolytic enzyme NE (9). Consequently, affected individuals develop emphysema by age 30 to 40, which results in a progressive respiratory impairment and a shortened life-span (15). Transfer of the normal α 1AT gene to lung cells has the potential to protect the lung from NE by local production of the functional antiprotease.

In CF, mutations of coding exons of the 250-kb CF gene are associated with abnormalities in respiratory epithelial cell secretion of thick mucus, chronic colonization of the epithelium with pathogens such as *Pseudomonas aeruginosa*, and airway inflammation dominated by neutrophils (14, 16). Because the Cl^- secretory abnormalities of epithelial cells with the CF genotype can be corrected by the transfer of the normal CF gene in vitro (17), it should be possible to overcome the expression of the abnormal gene by transfer of the normal gene to airway epithelial cells in vivo.

A recombinant adenovirus-ornithine transcarbamylase (OTC) vector administered intravenously to *spf-ash* mice (OTC-deficient) corrected the enzyme deficiency for at least 1 year (18), which suggests that long-term expression is possible. In the lung, long-term expression would be aided by stable integration of the transferred gene

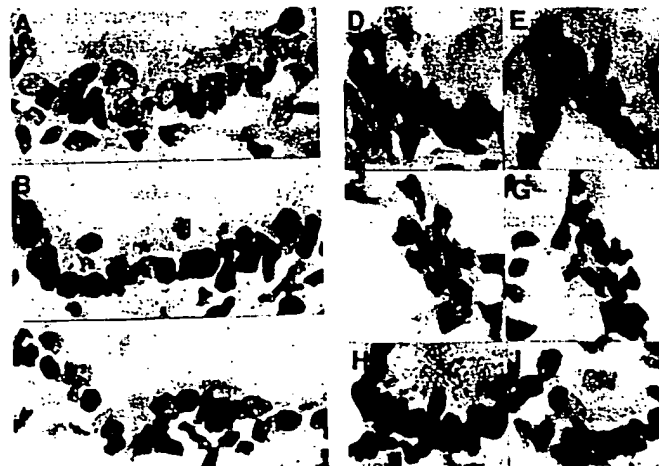
into the appropriate stem cells (1, 19, 20). Production of human α 1AT by lung cells continued for at least 1 week after in vivo infection with Ad- α 1AT. If an inability of the virus to infect stem cells limits the length of the time of expression, repetitive administration of the recombinant virus could be used, as long as safety is ensured.

Respiratory epithelial lining fluid (ELF) levels of α 1AT of 1.7 μ M are sufficient to protect the human lung from its burden of NE (21). Because the lavage fluid used to obtain the ELF diluted the ELF 50- to

100-fold (22), we estimate that the actual ELF levels achieved in experimental animals with a single infection of Ad- α 1AT were ~50-fold below threshold human protective level. Theoretically, it may be possible to achieve higher levels of α 1AT by increasing the viral titer, delivering Ad- α 1AT by aerosol (thus dispersing the vector over a broader surface area), and repeating the administrations of vector.

The safety aspects for human gene therapy of the recombinant adenoviral vectors, unlike retroviral vectors, have not been exam-

Fig. 4. In situ hybridization evaluation of lung from cotton rats infected in vivo with Ad- α 1AT. (A) Uninfected lung (PBS control) with antisense probe. (B through I) Several examples of Ad- α 1AT-infected lung. (B) Antisense probe. (C) As in (B) but with sense probe. (D) Antisense probe. (E) As in (D) but with sense probe. (F) Antisense probe. (G) As in (F) but with sense probe. (H) Antisense probe. (I) As in (H) but with sense probe. Cotton rats were infected in vivo as described in Fig. 3, with 300 μ l of PBS alone or with 300 μ l of Ad- α 1AT diluted to between 10^8 and 10^{10} PFU/ml in PBS. After 3 days, the lungs were exposed, blood was removed by cardiac puncture, and the lungs were lavaged. The trachea and pulmonary vasculature were perfused with 4% paraformaldehyde (PFA; Fluka Chemical Corp.); the lungs were resected, fixed in 4% PFA, and frozen. Cryostat sections (7 to 10 μ m) were serially treated with 0.2 M HCl and proteinase K (1 μ g/ml) immediately before hybridization and evaluated with 35 S-labeled antisense and sense RNA probes as described in Fig. 2. As far as was possible, serial sections were used for the antisense and sense probes. All cryostat sections were stained with HE (all panels $\times 515$).



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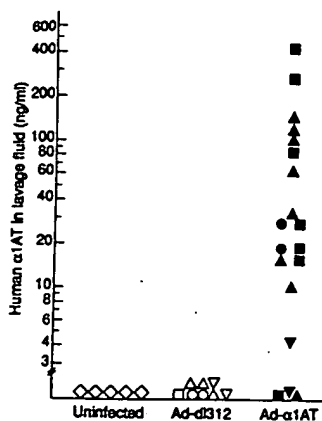


Fig. 5. The amount of human α 1AT in the respiratory ELF of cotton rats after in vivo infection with Ad- α 1AT. Animals were infected intratracheally with CsCl-purified Ad- α 1AT (10^8 to 10^{10} PFU/ml) as described in Fig. 3; controls included uninfected animals and those infected with a similar titer of Ad-d1312. From 1 to 7 days after infection, ELF was obtained by lavage of the lungs with 2 ml of PBS. Lavage fluid was clarified (700g, 20 min), and the concentration of human α 1AT was quantified (in quadruplicate) with a human α 1AT-specific ELISA (35) with a sensitivity of ≥ 3 ng/ml. Each symbol represents the mean value of an individual animal. All uninfected animals, \diamond ; for infected animals, 1 (\circ , \bullet), 2 (Δ , ∇), 3 (\square , \blacksquare), and 7 (\triangledown , \blacktriangledown) days after infection, respectively. No α 1AT was detected by ELISA in the viral preparations used for infection.

ined in detail. Safety is particularly important in weighing risk and benefit in response to α 1AT deficiency, in which augmentation therapy with human plasma α 1AT is available (21). In contrast, no definitive therapy is available for CF. Most human adults have antibodies to one of the three serogroup C adenoviruses to which Ad5 belongs (5). This implies little risk to those working with these vectors but may have negative implications for the virus as a gene transfer vector in the human lung. If such problems are encountered, alterations in the vector construct may be helpful. The encouraging results obtained with the Ad- α 1AT recombinant adenoviral vector in vivo suggest that similar recombinant vectors may be useful for in vivo experimental animal studies with genes such as the human CF gene.

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Group I Intron Self-Splicing with Adenosine: Evidence for a Single Nucleoside-Binding Site

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For self-splicing of *Tetrahymena* ribosomal RNA precursor, guanosine binding is required for 5' splice-site cleavage and exon ligation. Whether these two reactions use the same or different guanosine-binding sites has been debated. A double mutation in a previously identified guanosine-binding site within the intron resulted in preference for adenosine (or adenosine triphosphate) as the substrate for cleavage at the 5' splice site. However, splicing was blocked in the exon ligation step. Blockage was reversed by a change from guanine to adenine at the 3' splice site. These results indicate that a single determinant specifies nucleoside binding for both steps of splicing. Furthermore, it suggests that RNA could form an active site specific for adenosine triphosphate.

GROUP I INTRONS SHARE CONSERVED sequence elements and a common core secondary structure (1). Consistent with these similarities, there is a common mechanism by which group I introns are excised and the exons ligated (2, 3). A significant feature of all group I introns is the requirement for G (4) to initiate the splicing reaction (2, 5). The first step is a G-dependent cleavage at the 5' splice site. This step is a transesterification (phospho-

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ester transfer) reaction in which the substrate G is covalently joined to the 5' end of the intron. As a result, a free 3' hydroxyl group is generated on the U at the end of the 5' exon. In the second step of the splicing reaction, the exons are ligated by another transesterification reaction. In this case, attack occurs at the 3' side of the conserved G at the 3' end of the intron (G414) (6) (Fig. 1). This G is essential for completion of the splicing reaction (7). The 5' splice-site cleavage can be viewed as a "forward" reaction, and the exon ligation can be viewed as the "reverse" of the forward reaction (8-10). This idea was demon-

GENE 1380

Short Communications

Growth hormone gene expression in eukaryotic cells directed by the Rous sarcoma virus long terminal repeat or cytomegalovirus immediate-early promoter

(Recombinant DNA; rat GH₃ cells; retrovirus; enhancer; DEAE-dextran transfections)

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SUMMARY

The cytomegalovirus (CMV) immediate-early (IE) gene-regulatory region was found to be three- to fourfold more efficient than the Rous sarcoma retroviral long terminal repeat (LTR) in promoting expression of the bovine growth hormone (bGH) gene by rat GH₃ cells.

INTRODUCTION

Cis-acting DNA sequences found in the 5'-flanking regions or within eukaryotic genes are necessary for the promotion, enhancement and/or regulation of gene transcription (Benoist et al., 1980; Darnell, 1982; Gillies et al., 1983; Grosveld et al., 1982; Minty and Newmark, 1980). Eukaryotic viruses have been shown to possess enhancer/promoter sequences which are important in viral gene expres-

sion (Benoist and Chambon, 1981; Temin, 1982; Yaniv, 1982). Retroviral LTRs contain control sequences which direct the synthesis of progeny viral RNA and viral mRNAs (Temin, 1982). The human CMV genome contains a major IE gene, located in the long unique region of the viral genome (Stenberg et al., 1984; Stinski et al., 1983) which is expressed in large amounts after infection (Wathen and Stinski, 1982).

We were interested in comparing the RSV LTR and the CMV IE promoters in their ability to direct eukaryotic gene expression in a similar host-cell environment. Recombinant plasmids were constructed which contain viral promoters ligated to the coding sequences of the bGH gene. Using a eukaryotic assay system previously described (Kopchick et al., 1984; 1985), we compared the two promoters in their ability to direct synthesis of bGH.

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bGH, bovine growth hormone; bp, base pairs; CMV, cytomegalovirus; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle medium; IE, immediate-early region; kb, 1000 bp; LTR, long terminal repeat; nt, nucleotide(s); PA, polyacrylamide; RSV, Rous sarcoma virus; Tc, tetracycline.

EXPERIMENTAL AND DISCUSSION

(a) Construction of bGH expression vectors

Recombinant plasmid DNAs termed pBGH-4 and pCMVIE-BGH (Fig. 1) were used in this study. Both constructions were derived from pBGH-2, a plasmid which contains a *Bam*HI-*Eco*RI fragment derived from the bGH gene (Kopchick et al., 1985). This DNA fragment encodes the 5' mRNA non-translated leader sequence, five exons, four intervening sequences, and the 3'-flanking untranslated region found in the bGH gene (Woychick et al., 1982). We previously reported that the 5'-noncoding sequences found in the native bGH gene (clone pBGH-1) were inactive in directing detectable levels of bGH synthesis and secretion in rat GH₃ cells (Kopchick et al., 1985).

The cloning strategy of pBGH-4 has already been described (Kopchick et al., 1985). Briefly, pBGH-4 contains an approx. 1.5-kb DNA fragment isolated from a plasmid clone of Schmidt Rupp B strain of RSV. This fragment includes the retroviral LTR

which has been ligated to the 5' end of the bGH coding sequences (Fig. 1).

Plasmid pCMVIE-BGH (Fig. 1) was derived from the recombinant plasmid p16T-132 (M.J.T., unpublished). A 2-kb *Pst*I fragment isolated from a CMV (Eisenhardt strain) genomic clone bank was subcloned in pBR322 and was termed p16T-132. It encodes a promoter-regulatory region similar to that of the Towne strain of CMV (Thomsen et al., 1984; M.J.T., unpublished). Plasmid pCMVIE-*Bgl*II was obtained from p16T-132 by introducing an 8-bp *Bgl*II linker at a unique *Sac*II restriction site located in the 5' untranslated leader sequence of the CMV major immediate early mRNA (Stenberg et al., 1984). The pCMVIE-BGH cloning procedure was performed as follows. Two DNA fragments were generated by digesting pBGH-2 with *Bam*HI + *Eco*RI. Digestion of pCMVIE-*Bgl*II with *Eco*RI + *Bgl*II also resulted in two DNA fragments. Equimolar concentrations of the four DNA fragments were mixed and ligated with the T4 DNA ligase (IBI, New Haven, CT). Recombinant DNA plasmids were used for transformation of *Escherichia*

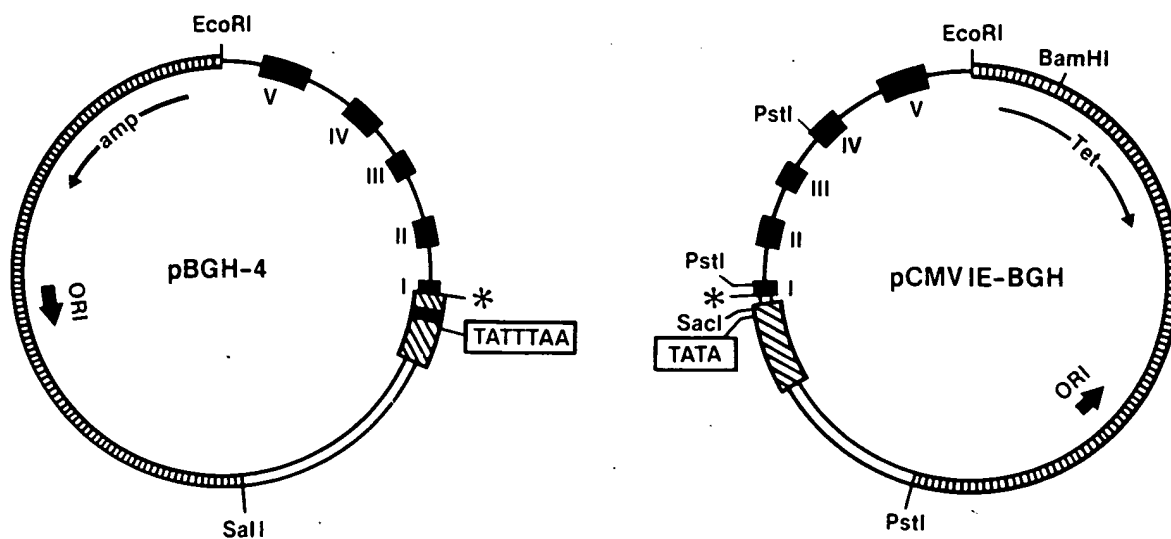


Fig. 1. Comparison of the recombinant plasmids pBGH-4 and pCMVIE-BGH. Each construct contains a *Bam*HI-*Eco*RI restriction fragment (approx. 1.9 kb) encoding the five exons (closed boxes, I, II, III, IV, V), four introns, and 3'-untranslated flanking sequence (thin line) of the bGH gene cloned in pBR322 (double striped lines). pBGH-4 contains a *Sal*I-*Bst*EII restriction fragment (approx. 1.5 kb) derived from a plasmid clone of a Schmidt Rupp B strain of RSV (pL397) and includes part of the retroviral *env* gene (double open lines) and the retroviral LTR (hatched box) ligated to the 5' end of the bGH between the *Bam*HI and the *Bst*EII restriction termini (asterisk) as previously described (Kopchick et al., 1985). The location of the putative Hogness box (TATTTAA) is indicated. pCMVIE-BGH contains a *Pst*I-*Bgl*II restriction fragment (approx. 1.1 kb) isolated from a plasmid clone (pCMVIE-*Bgl*II) of the Eisenhardt strain of human CMV (double open lines). This fragment includes the promoter-regulatory region (hatched box) of the major IE gene, linked to the 5' end of the bGH structural gene between the *Bam*HI and the *Bgl*II termini (asterisk). The location of the TATA box is indicated.

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coli RR1 cells and transformants were selected for Tc resistance. DNA from bacterial colonies that hybridized to a bGH probe were isolated and analyzed by restriction endonuclease digestion. A recombinant plasmid termed pCMVIE-BGH was selected and the DNA purified. This plasmid contains the bGH coding gene sequences attached to the promoter region of the major IE gene from the Eisenhardt strain of human CMV (Fig. 1).

(b) Nucleotide sequencing

The nucleotide sequence of the hybrid *Bg/II-B*: *mHI* site at the junction of the CMV and bGH

fragments and the structure of the CMV IE promoter are shown in Fig. 2A (Thomsen et al., 1984). For comparison, the structure of the RSV LTR is depicted in Fig. 2B; the exact nucleotide sequence at the junction of the avian retrovirus and the bGH fragments was analyzed previously (Kopchick et al., 1985). It is assumed that transcription initiates from the normal cap site found within the LTR and CMV promoters and continues into the nearby bGH sequences. The locations of the TATA box, the CAAT box and the cap site are shown (Fig. 2, A and B). A consensus promoter sequence (TATTTAA) has been located in the U3 region of the LTR, 30 nt upstream from the viral RNA start point found in the

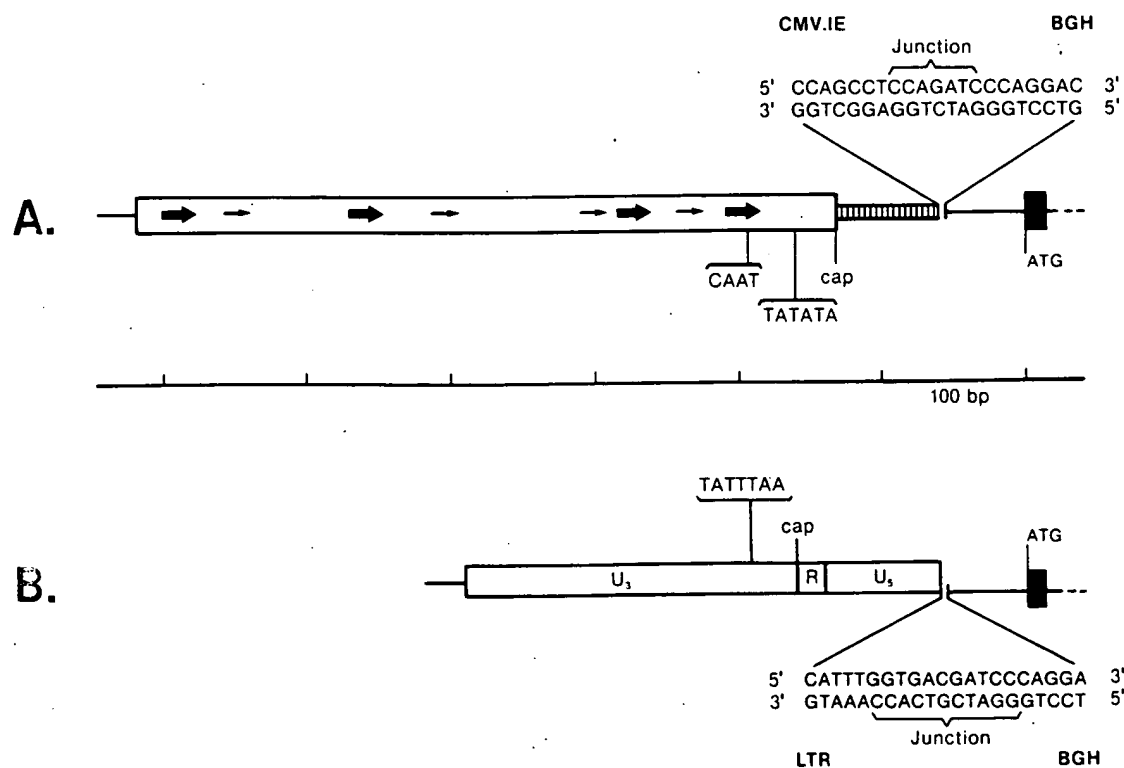


Fig. 2. Nucleotide sequence of the CMVIE-BGH and RSV-BGH junctions. (A) pCMVIE-BGH. The structure of the CMV IE-promoter and its junction with the first exon of the bGH gene is summarized. The relative position of the viral mRNA cap site (cap), the TATA and CAAT regions, and of the 19-bp (thick arrows) and 18-bp (thin arrows) direct repeat sequences are shown, as previously reported (Thomsen et al., 1984). The exact nucleotide sequence at the junction between the viral (CMV IE) and bovine (bGH) DNA fragments was determined. Briefly, pCMVIE-BGH was cleaved with *Pst*I. A 1.2-kb DNA fragment containing the CMV sequence and a portion of the first exon of the bGH gene (Fig. 1) was isolated from a 1% low melting agarose gel (Seaplaque from FMC, Marine Colloid Division, Rockland, MA). The *Pst*I fragment was 3' end labeled with dideoxyadenosine 5'-[γ - 32 P] triphosphate (ddATP, 250 μ Ci, approx. 300 Ci/mmol; Amersham) and terminal transferase. Fragments with a single 3' -labeled end were produced by digesting the *Pst*I fragment with *Sac*I. The *Sac*I-*Pst*I fragments (approx. 133 bp) were separated on an 8% PA gel, purified and sequenced (Maxam and Gilbert, 1980). (B) pBGH-4. The structure of the RSV LTR is summarized: U3, portion of the LTR derived from the 3' terminus of viral RNA; U5, portion of the LTR derived from the 5' terminus of the retroviral RNA; R, 21-bp terminal redundancy found in viral genomic RNA. The viral mRNA cap site (cap) and Hogness box (TATTTAA) are shown (Schwartz et al., 1983). The strategy used for sequencing the junction between the RSV promoter (LTR) and the bovine genomic DNA (bGH) has been described (Kopchick et al., 1985). The position of the translation start codon (ATG) in the first exon of bGH is indicated (Woychick et al., 1982).

R region of the LTR (Fig. 2B) (Ju and Skalka, 1980; Schwartz et al., 1983). Typical TATA and CAAT boxes (Fig. 2A) are found 28 bp and 61 bp, respectively, upstream of the cap site, in the CMV IE promoter (Thomsen et al., 1984). The 5'-mRNA non-translated leader sequences, extending from the viral RNA cap site to the bGH ATG translational start codon, are 164 bp and 128 bp long, for pBGH-4 and pCMVIE-BGH, respectively.

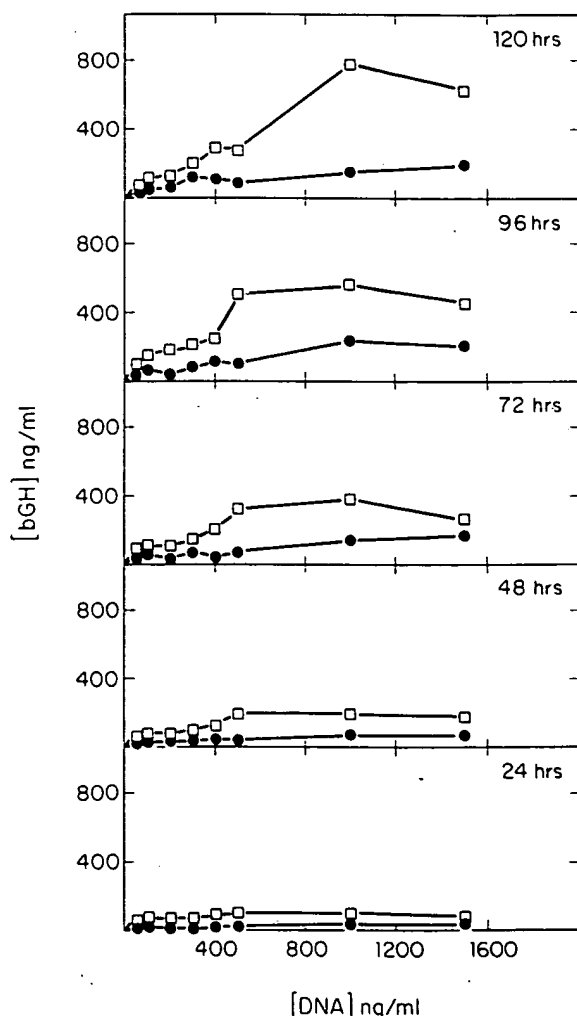


Fig. 3. Bovine growth hormone expression by transiently transformed GH₃ cells. Equal amounts (ranging from 50 to 1500 μ g/ml) of purified pBGH-4 or pCMVIE-BGH DNAs were transfected into rat GH₃ cells in the presence of DEAE-dextran (200 μ g/ml). Secretion of bGH into the culture fluid was assayed by a standard double antibody radioimmunoassay (Leung et al., 1984). The production of bGH by the cells transformed with pBGH-4 (●—●) or with pCMVIE-BGH (□—□) was measured 24, 48, 72, 96, and 120 h after transfection and is expressed in ng/ml of culture medium (DMEM). A total of 2 ml of culture fluid was used in each cellular incubation.

(c) Production of bGH

pBGH-4 and pCMVIE-BGH plasmid DNAs were introduced transiently into cultured rat GH₃ cells. The concentration of DNA that resulted in optimum bGH expression was between 500 to 1000 ng/ml (Fig. 3) and is in agreement with DNA concentrations reported previously for other cell types (Kopchick and Stacey, 1984). The time course of the bGH expression directed by the optimum level of pBGH-4 or pCMVIE-BGH DNAs in rat GH₃ cells is shown in Fig. 4. Synthesis of bGH begins between 1 and 2 days post-transfection and increases regularly for up to 5 days. Transfected cells continue to secrete bGH for up to 14 days post-transfection with optimal bGH levels detected between days 5 and 9 (Fig. 4). This time course is in agreement with results previously reported in transient gene expression for other cell lines (Kopchick and Stacey, 1984). At 24 h post-transfection, cells transfected with pBGH-4 did not secrete detectable levels of bGH but cells transfected with pCMVIE-BGH did produce bGH (75 ng/ml). The difference in bGH secretion at 24 h is statistically significant ($P < 0.001$, Student's *t*-test). The synthesis of bGH by GH₃ cells transfected with pBGH-4 DNA increased during the first 3 days post-transfection but never surpassed 230 ng/ml. However, bGH secretion by cells transfected with

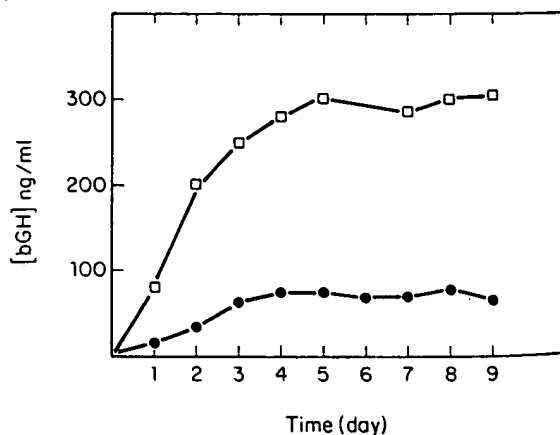


Fig. 4. Bovine growth hormone secretion at different timepoints posttransfection. The production of bGH by the rat GH₃ cells transfected with pBGH-4 (400 ng/ml; ●—●) or pCMVIE-BGH (400 ng/ml; □—□) was assayed daily (see legend of Fig. 3) for 9 days posttransfection and is expressed in ng/ml of culture medium (DMEM). A total of 2 ml of culture fluid was used in each cellular incubation.

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Expression of human α_1 -antitrypsin using a recombinant adenovirus vector

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In order to improve the *in vivo* delivery of human proteins destined for replacement therapy, a recombinant adenovirus containing the cDNA sequence encoding human α_1 -antitrypsin has been constructed and shown to direct expression of the protein in the supernatant of human cell lines.

Antiprotease; Serine protease; α_1 -Antitrypsin; Gene targeting; Drug delivery, *in vivo*; Recombinant adenovirus

1. INTRODUCTION

α_1 -Antitrypsin (α_1 AT) is a serum antiprotease which is mainly produced by hepatocytes and mononuclear phagocytes and whose major physiological function is to inhibit neutrophil elastase (NE) activity in the lung [1]. NE is a serine protease capable of degrading proteins of the alveolar interstitium, the result of this action being the destruction of the lung parenchyma [2]. Severe hereditary deficiency of α_1 AT is associated with low plasma and lung levels of α_1 AT and predisposes affected individuals to pulmonary emphysema by impairing the protease/antiprotease balance in the lower respiratory tract [3,4].

α_1 AT replacement is currently being assessed in the therapy of hereditary emphysema [5]. Large quantities of recombinant unglycosylated α_1 AT have been made available by production in *E. coli* or yeast but the absence of sugar side chains results in a shorter plasma half-life [6-8].

To circumvent this problem the product could be delivered directly to the site where the pathology of the deficiency is manifest, i.e. the alveolar epithelium. Aerosol administration of recombinant α_1 AT has been shown to be effective in α_1 AT-deficient patients [8,9]. Another possible approach is to implant in the lung autologous cells secreting 'normal' α_1 AT or to deliver the correct α_1 AT gene *in situ* via, for instance, a recombinant viral vehicle infecting the lung epithelial cells.

Both approaches rely on an efficient vector suited for high level and stable expression of α_1 AT in exogenous cells.

Retroviral vectors have recently been used to produce glycosylated recombinant α_1 AT [10]. Transformed fibroblasts have been implanted in the peritoneal cavity of nude mice and human α_1 AT secreted by these cells has been detected in plasma and in the epithelial fluid of the lungs [11]. Although confirming the feasibility of the strategy, these experiments, however, demonstrated the need for improvement in the technology since the amounts of α_1 AT produced by the retrovirus-infected cells were low.

2. EXPERIMENTAL

2.1. Construction of the expression plasmid MLP- α_1 AT

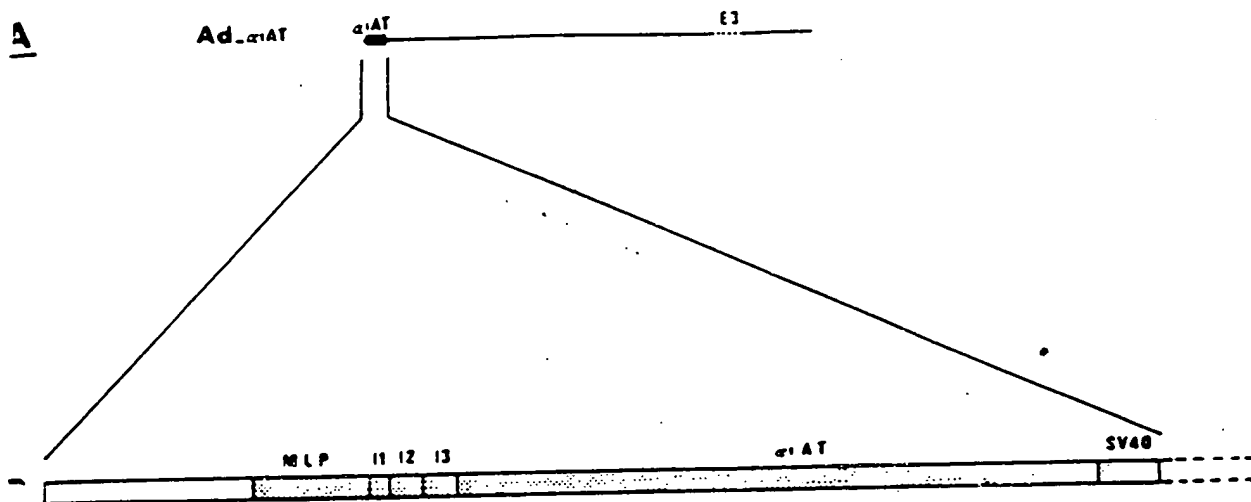
Plasmid MLP- α_1 AT contains the following sequences inserted between the *EcoRI* and *BamHI* restriction sites of pBR322. (i) The 455 base pairs of the left end of the human adenovirus (Ad) type 5 genome (nucleotides 1-455). This fragment carries the Inverted Terminal Repeat, the encapsidation signal sequences and the enhancer of the E1A promoter. (ii) The major late promoter (MLP) of the human Ad type 2 (nucleotides 5780-6038), followed by an almost complete copy of the tripartite leader cDNA sequence (nucleotides 6039-6079; 7100-7171; 9634-9713). (iii) The complete human α_1 AT cDNA and (iv) the polyadenylation signal from the SV40 A gene (nucleotides 2666-2533).

2.2. Construction of recombinant adenovirus

The plasmid MLP- α_1 AT was digested with *PstI* and *AccI* within the pBR322 sequence, and the excised fragment was cloned into the unique *Clal* site (map units 2.6) of Ad type 5, thus replacing a portion of the viral E1A gene (Fig. 1). To obtain an acceptable DNA size for encapsidation, it was necessary to use an Ad deleted in the E3 region (d1327), which is not essential for the growth of the virus *in vitro* or *in vivo*. After ligation, the DNA was used to transfect Ad type 5-transformed human 293 cells [12], using calcium phosphate precipitation [13]. A recombinant adenovirus- α_1 AT (Ad- α_1 AT) was isolated after 7 days, as shown by DNA analysis.

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(A) Structure of the recombinant adenovirus (Ad- α_1 AT): the Ad5 d1327 derivative, which has the E3 region deleted, was used as vector for the chimeric α_1 AT transcription unit. (B) Enlarged is the left end of the recombinant genome containing the chimeric α_1 AT transcription unit: sequences corresponding to the major late promoter (MLP) and to the tripartite leader (11, 12, 13) have been used to drive the transcription of the α_1 AT cDNA and the SV40 mRNA processing signals.

3. α_1 AT expression

α_1 AT expression was monitored by ELISA essentially as described [14]. Goat anti-human α_1 AT (Cappel, Melvern, PA, USA) was used as the primary and secondary antibody, the latter being tagged with peroxidase.

RESULTS AND DISCUSSION

Transient expression of α_1 AT was tested in CHO cells after transfection with plasmid MLP- α_1 AT. At 15 h post-transfection, 2.5 μ g/ml of α_1 AT were found in the supernatant (not shown). This demonstrated that the MLP directs the transcription of the α_1 AT cDNA to an mRNA that is efficiently translated.

Expression levels of α_1 AT synthesized after Ad- α_1 AT infection of 293 and HeLa cells are presented in Table I. Infection by the recombinant adenovirus at a multiplicity of 10 or 100 p.f.u. per cell led to accumulation of α_1 AT in the medium. After 6 days of infection with 100 p.f.u. of HeLa cells 60 μ g/ml of α_1 AT were detected. The results show that Ad can be used in vitro

to express high levels of human α_1 AT. Amounts are higher for HeLa cells (which do not complement the E1A defect) than for 293 cells where the recombinant virus can grow efficiently. This observation can be explained by the fact that in HeLa cells the cellular machinery can transcribe the viral genes more efficiently as it is less compromised by the growth of the virus.

4. CONCLUSION AND PROSPECTS

With the goal of improving the expression of recombinant glycosylated α_1 AT we have explored the use of an Ad vector. Ad vectors have proved useful for achieving high-level expression of a variety of foreign genes in different cell types [15-19]. Ad infection in humans is benign and no neoplastic transformation has been associated. Here we report the construction of a recombinant Ad bearing the cDNA of the human α_1 AT under the control of the viral major late promoter. Since the E1A region is replaced, this vector is replication-defective unless propagated in human 293 cells which complement the E1A defect, but can infect a variety of cell types where stable genomic integration of the viral sequences can occur.

Our results argue in favor of the possibility of a recombinant adenovirus vector being used to correct in vivo α_1 AT deficiency.

Table I

Time course of extracellular α_1 AT production

Day	293 cells		HeLa cells	
	10 p.f.u.	100 p.f.u.	10 p.f.u.	100 p.f.u.
1.933	3.937		0	0.625
3.750	5.400		0.325	4.375
4.250	5.050		3.800	41.250
4.250	5.500		31.250	60.000

The cumulative amounts of human α_1 -antitrypsin (expressed in μ g/ml) produced after infection of 1×10^7 293 and HeLa cells with 10 or 100 p.f.u. of Ad- α_1 AT virus

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pCMVIE-BGH DNA increased from approx. 75 ng/ml at 24 h post-transfection to 760 ng/ml at 120 h.

Thus, the CMV IE promoter appeared more efficient than the RSV LTR in its ability to direct expression of the bGH gene in GH₃ cells at all times posttransfection. Also pCMVIE-BGH plasmid DNA led to the synthesis of three to five times more bGH than did pBGH-4 DNA, independent of the dose of transfected DNA (Fig. 3) or time post-transfection (Fig. 4). Similar results were obtained in transiently transfected mouse L cells (not shown).

Since the hybrid DNA molecules, RSV-BGH and CMVIE-BGH, were cloned into pBR322 at different restriction sites and in different orientations, a possibility exists that plasmid sequences may influence transcription of the bGH gene. DNAs encoding the hybrid genes were excised from the pBR322 vector and purified by gel electrophoresis. Transfection of the linear purified DNA fragments into GH₃ cells yield results identical to those presented above, indicating that pBR322 nucleotide sequences do not influence transcription of the bGH gene in either of the hybrid molecules (not shown). The transfection efficiencies of linear vs. circular DNA molecules are similar to those reported previously (Kopchick and Stacey, 1984).

(d) Comparison of promoters

The molecular characteristics that enable the CMV IE-promoter/enhancer to be more efficient than the RSV LTR or the natural bGH 5' regulatory flanking sequences in directing expression of bGH in GH₃ cells are unknown. The precise locations of the TATA box, the CAAT box and the viral mRNA cap site in the CMV IE promoter-regulatory region have been defined (Thomsen et al., 1984). Sequence analysis of the human CMV IE promoter revealed nucleotide repeating units of 16, 18, and 19 bp (Thomsen et al., 1984). The 19-bp repeats appear highly conserved between different strains of human and simian CMV. Thomsen et al. (1984) suggested a correlation between these repeat sequences, the formation of cruciform structures, and the CMV IE-promoter strength. In the case of retroviruses, viral RNA synthesis has been shown to initiate at the cap site in the R region using promoter sequences located in U3 (Temin, 1982); a putative Hogness box ap-

peared 30 nt upstream from the cap site in the RSV genomic DNA sequence (Ju and Skalka, 1980; Schwartz et al., 1983). A functional CAAT box was described in the U3 region of the Abelson murine leukemia virus (Srinivasan et al., 1984) but no homologous transcriptional regulation signal appeared in the RSV LTR sequence (Schwartz et al., 1983). The RSV LTR has been shown to contain an efficient promoter as well as an enhancer (Laimins et al., 1984; Luciw et al., 1983; Skalka et al., 1983). Short sequences in the RSV LTR were found homologous to the tandem repeat enhancer element of SV40 (Ju and Skalka, 1980; Weiher et al., 1983). Enhancers have also been identified within the U3 region of various retroviral LTRs (Khoury and Gruss, 1983; Srinivasan et al., 1984), and different studies suggested a correlation between the LTR enhancer activity and the activation of cellular protooncogenes by retroviruses (Khoury and Gruss, 1983; Temin, 1982). Further experiments that explicitly compare the role of these particular sequences in the LTR and CMV IE-promoter/enhancer are now required.

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